UNDERSTANDING THE SPECIFICITY OF TETRACYCLINE RECOGNITION BY A PUTATIVE RNA TOXIN SENSOR: THE YKKCD RIBOSWITCH

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What are antibiotics and how are they affecting people in the United States?

The first person to use the word antibiotic was Selman Waksman. He used it to describe any small molecule that was produced by a microbe and prevented the growth of other microbes. Thus many antibiotics such as penicillin, streptomycin, and tetracycline came to be. All three were developed between 1945 and 1955. One main difference between these antibiotics is how they are produced. A fungus produces penicillin, while soil bacteria produce streptomycin and tetracycline (1). There are currently 160 classes of antibiotics known to date, most being discovered between 1940 and 1960 (2). Although antibiotics have been used to treat infections, the problem is that many antibiotics first discovered have become ineffective due to the evolution of antibiotic resistance by human pathogens.

In the United States alone, hospital-acquired infections afflict nearly 2 million people, and of the 2 million people affected, approximately 100,000 people will die per year (3). The reason for the high numbers may be due to the fact that 70% of the infections are resistant to at least one antibiotic. The number of antibiotic resistant strains has increased at a rapid rate. The number of antibiotic

resistant strains in San Francisco County were almost doubled and sometimes tripled between 1997 and 1998 (CDC). Since this time the numbers have increase 10-20% (4). This was an indication of a trend occurring nationwide (see Figure 1). As a result the CDC estimates the direct costs associated with hospital infections are as high as $45 billion dollars each year. Justifiably we can say that antibiotic resistance is a severe problem.

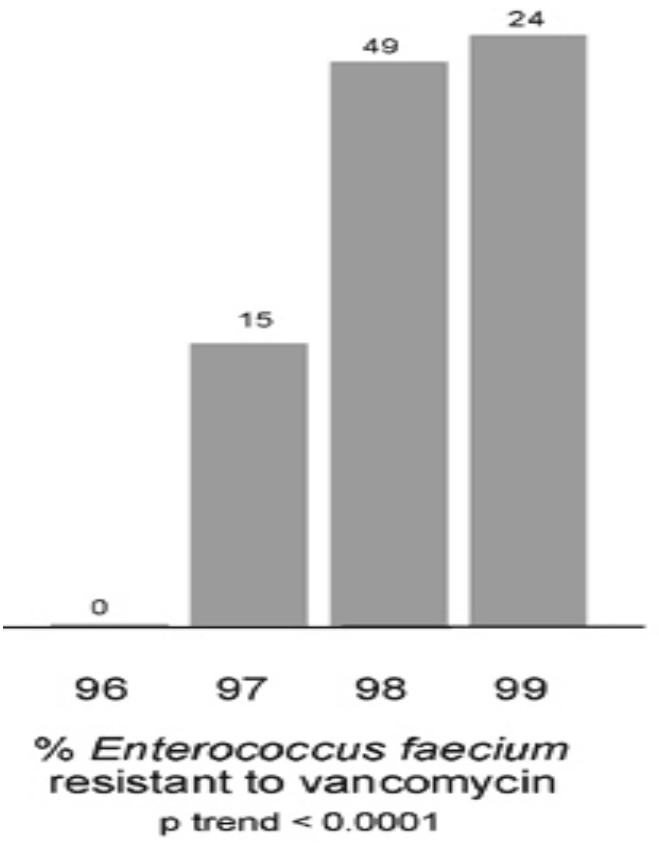


Figure 1. Representation of the increase in antibiotic resistance from 1996 to 1999.

Communication in Bacteria

For many years it was believed bacteria were populations of cells that worked independently. However it has been discovered recently that bacteria interact and communicate with each other. In fact bacteria produce secondary metabolites that respond to various chemicals in their environment. Some secondary metabolite groups have been identified as playing a part in the regulation of gene expression in a cell-density-dependent manner known as quorum sensing or cell-cell communication (5). It is believed that this results from the formation and accumulation of signaling molecules. These signaling molecules, known as autoinducers, will lead to gene expression changes when they are bound to receptors on or in the bacterial cell at a threshold concentration.

There is no clear-cut explanation of how common it is for cell-cell signaling to occur between bacteria cells. However there are two main components of communication to take into consideration: the cost associated with signaling and the specificity of the information (5). The problem is the signal produced and the mechanism necessary to bring about a response is not clear. For proteins, production and export of signals acts catalytically (5). For this reason it is

assumed that the cost is of top priority. It is believed that production is inversely proportional to production cost. With specificity it is about the amount of information going into the signal and seems to correlate to the cost of production (5). Bacteria, especially in the soil, form antibiotics. However, they are only produced when they are needed because they are costly to the cell. There are few pathogens in the soil, which led scientists to believe that antibiotics are not only being produced as killers, but as signaling molecules in the cell.

(1) What is antibiotic resistance?

Antibiotics are not just bacterial weapons that fight competitors; they are also signaling molecules that can regulate the homeostasis of microbial communities. In fact antibiotics are hormesis. Hormesis is a concept that was originally used to describe the effects of low doses of radiation as a means of killing molecules (6). A broader usage of the term is to describe biological responses to environmental signals and stresses, which act as stimulants at lower doses and as inhibitors at higher doses. In much the same way antibiotics act as bacterial killers at a high concentration but can produce changes that help bacteria in nature at lower concentrations.

The first issue to consider is how a bacterium becomes resistant to the antibiotics. Bacteria that carry an antibiotic resistance gene in the plasmid of the bacteria cell can become resistant to antibiotics. This gene can easily be transferred

to the plasmid of another bacteria cell by horizontal gene transmission (Figure 2). Bacteria can modify, degrade the antibiotics, or with the help of an efflux pump, remove the antibiotics out of the cell, thus rendering the antibiotics inactive (Figure 3). These defense mechanisms become active when toxin sensors in the bacterium modify or detect antibiotics in the cell. The toxin sensor can trigger protein production that modifies or degrades the antibiotics or triggers an efflux pump to pump the antibiotics out of the cell. The efflux pump will only be produced when it is needed.

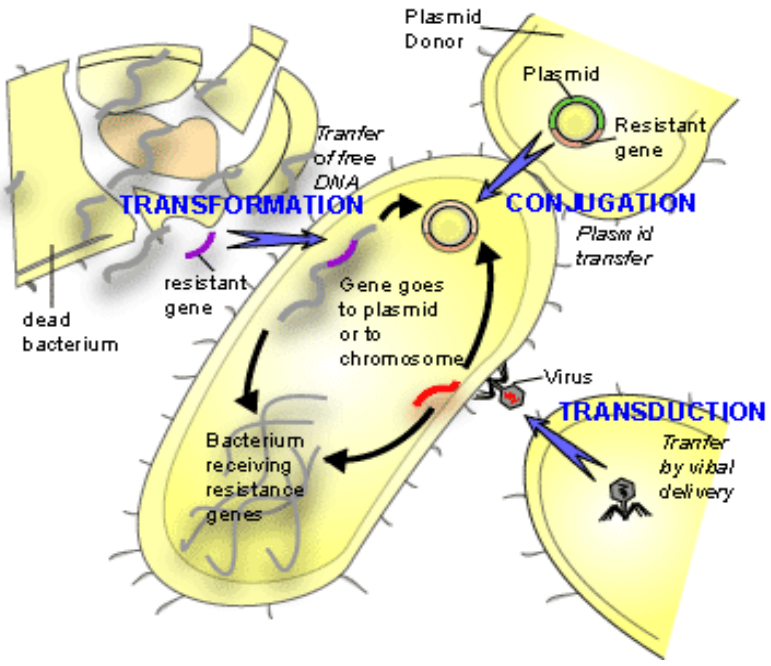


Figure 2. This figure is indicating bacteria actively transferring the resistance gene, which will lead to another bacterium becoming resistant to antibiotics.

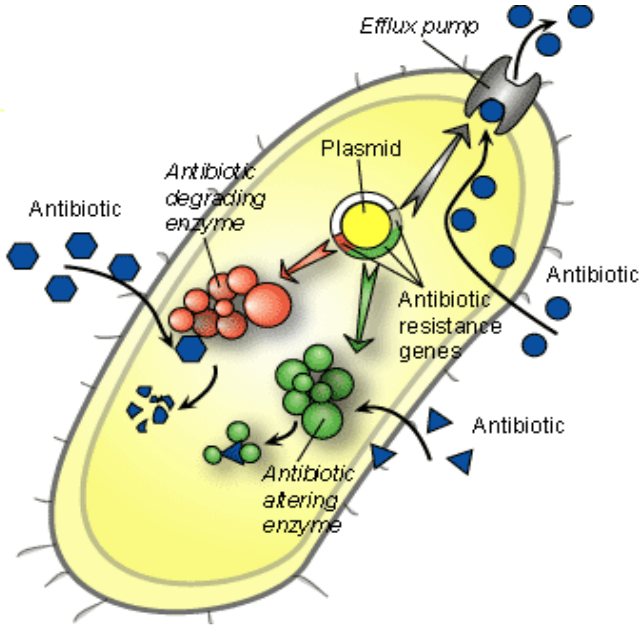


Figure 3. The efflux pump was triggered by the toxin sensor in the bacterial cell to transport the antibiotics from the cell and into the external environment.

The toxin sensor works like a light switch: it only turns on and off. When no antibiotics are present, the toxin sensor is off, and the efflux pump is not produced. However when the antibiotic concentration reaches its threshold, the toxin sensor recognizes the antibiotic and triggers production of the efflux pump by gene expression regulation. Gene expression regulation by riboswitch RNA sensors is dependent upon an allosteric conformational change that is triggered by binding of a small molecule (the antibiotic) to the mRNA. When the antibiotic is not bound, then the resistance gene is not produced. Thus by inactivating the sensor we can prevent resistance gene production can be prevented.

(a) **Transcription**

To get a deeper understanding of the mode of regulation we have to discuss the process of transcription and translation. “Transcription is the process in which single stranded RNA with a base sequence complementary to the template strand of DNA is synthesized” (7). Transcription generates several types of RNA. These include mRNA, tRNA, and rRNA. In bacteria one RNA polymerase catalyzes the synthesis of all RNAs.

Transcription consists of three steps: initiation, elongation, and termination. The initiation of transcription involves the binding of RNA polymerase to a promoter. A promoter is a regulatory DNA sequence located upstream from the gene. The sigma factor recognizes promoters. They are promoter specific. RNA polymerase uses different sigma factors. After the sigma factor has fallen off and the affinity of the RNA polymerase complex has decreased for the promoter site, elongation can begin. During this phase the RNA polymerase converts to an active transcription complex as it binds numerous proteins. Ribonucleotides are continuously added until a termination signal is made (Figure 4). In bacteria there are two types of termination, intrinsic termination also known as rho-independent termination, and rho-dependent termination. Intrinsic termination comes about by transcription of the termination sequence that consists of an inverted repeat sequence followed by 6-8 adenines. The terminator stem-loop directs RNA polymerase and will act as a roadblock to stall the polymerase (8). The RNA polymerase will simulate the release of the freshly made mRNA by rho-independent

terminators (9). It is released because there are only weak A-U base pairs following the terminator stem. Rho-dependent termination comes about with the aid of the rho factor. This ATP-dependent helicase binds to a specific recognition sequence on the mRNA strand up stream from the terminator site and then unwinds the RNA-DNA helix to release the mRNA and stop transcription.

Riboswitches are RNA based control mechanisms, which can potentially repress active gene expression by triggering transcription or translation. Subinhibitory concentrations cause significant transcription modulation.

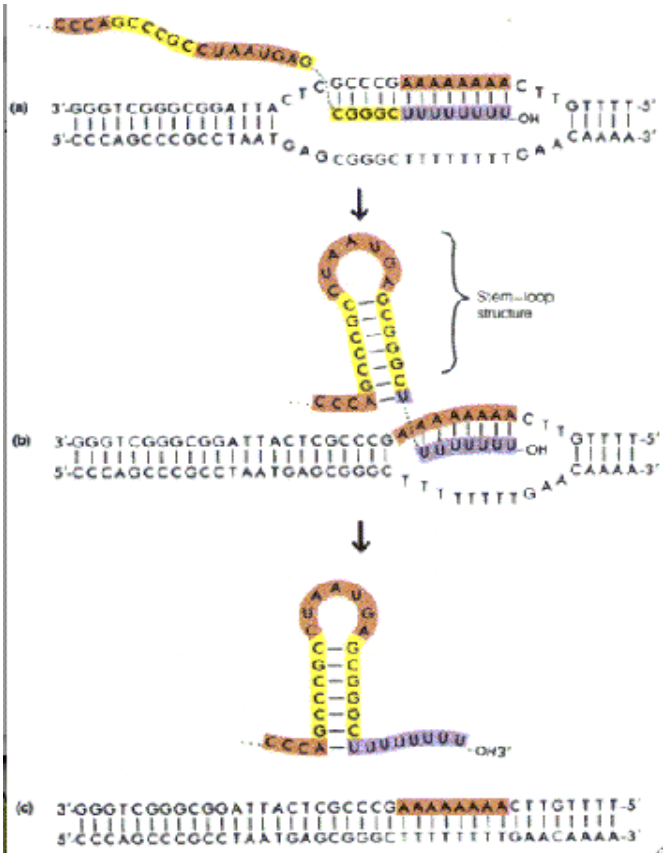


Figure 4. Transcription mechanism.

(b) Translation

Protein synthesis is regulated by the 5′ untranslated region (UTR) of the mRNA transcript (10). The ribosome binding site (RBS) binds to the ribosome to set up the correct sequences that will initiate translation. "The RBS controls the accuracy and efficiency with which the translation of mRNA begins". (11). It is called the Shine-Dalgarno sequence. Protein synthesis is, "the process by which the genetic message carried by mRNAs directs the synthesis of polypeptides with the aid of ribosomes and other cell constituents" (7). The translation of a genetic message into a primary sequence of polypeptides is done in three steps similar to that of transcription: initiation, elongation, and termination. Translation starts with initiation, which occurs once the small ribosomal subunit, binds an mRNA. The initiator tRNA base pairs with the initiation codon AUG on the mRNA and ends once the large ribosomal subunit combines with the small subunit. There are two sites for the codon-anticodon interaction on the ribosome. One being the P (peptidyl) site and the other is the A (aminoacyl) site, in the elongation phase. The polypeptide is synthesized, based on the information received from the genetic message (or codon sequence). The mRNA base sequence is read in the 5′ to 3′ direction and the polypeptide sequence starts at the N-terminal and ends at the C-terminal. The elongation process starts when a second aminoacyl-tRNA is attached to the ribosome in the A site due to codon-anticodon base pairing. Peptidyl transferase catalyzes the peptide bonds formed. For this to occur the α-amino group of the A site amino acid attacks the carbonyl group of the P site amino acid. Ribosomes move along the mRNA and

the next codon enters the A site. The growing peptide chain then moves to the P site. This is the elongation process that is continued until a stop codon enters the A site. Termination occurs when the polypeptide chain is released from the ribosome. This process comes about because a stop codon cannot bind an aminoacyl-tRNA. Thus a protein releasing factor binds to the A site. The peptidyl transferase hydrolyzes the completed polypeptide chain and the tRNA in the P site. Translation ends as the ribosome releases the mRNA and dissociates into the large and small subunits (Figure 5).

Transcription and translation can be modulated in many different ways in bacteria. Most antibiotics work as inhibitors of translation. The ribosome has receptors for many natural product inhibitors. All steps of translation on prokaryotic ribosomes are targets for specific antibiotic inhibitors (6). While this is true, there are very few ribosomal inhibitors of eukaryotic translation that are known. The difference between bacterial and eukaryotic processes has to be explored because the antibiotic cannot attack the host but it must attack the pathogen. Thus the difference between prokaryotic and eukaryotic translation have to be exploited so that only bacterial translation is affected. Bacterial translation is quite prone to mutagenis thus rendering the antibiotics useless.

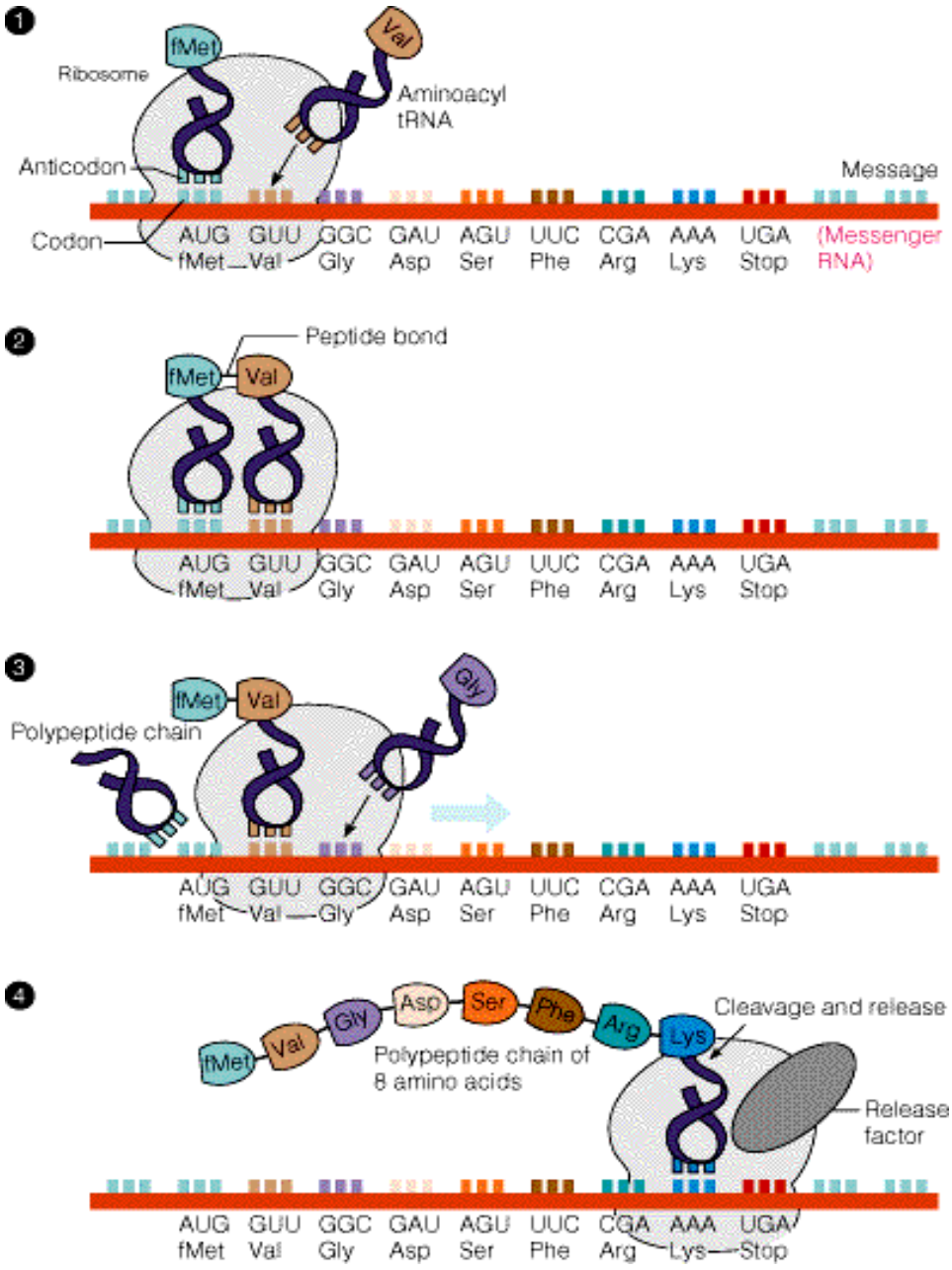


Figure 5. Translation mechanism.

The riboswitch is considered a new class of regulatory RNAs. What makes them so special is the fact that they do not require any intermediary sensory molecules. They behave as small sensors of small molecules themselves. These

natural RNA aptamers seem to control expression of numerous metabolic genes in bacteria and possibly in higher organisms. They are made more commonly in bacteria. These riboswitch aptamers are well conserved and appear not to change during evolution. Thus they are expected to be less prone to resistance causing mutagenesis.

(2) How do riboswitches regulate gene expression?

The expression of many genes necessary to metabolite biosynthesis or transport in bacteria is regulated by mRNA structures known as riboswitches. They are mostly found in the 5′ untranslated region of the mRNA of the target gene. Genes that contain riboswitches code for proteins that are involved in transport or synthesis of molecules that are costly to produce such as thiamine pyrophosphate (TPP) or flavin mononucleotide (FMN). They work as feedback inhibitors. There have been 12 different classes of riboswitches reported (Figure 6).

Each class of riboswitches forms a structured receptor, known as an aptamer, which binds to a specific fundamental metabolite without the help of a protein factor as well as a gene expression regulator known as the expression platform. Riboswitches within each class bind to the same metabolite and share highly conserved sequences and secondary structures. This indicates that all riboswitches of a given class form a common fold to recognize their cognate ligand (12). "Additionally, some bacterial genomes carry multiple riboswitches of the same class,

each regulating a different operon" (12). Many antibacterial drugs can be more effective due to many of the same classes of riboswitches being found in numerous different bacterial species.

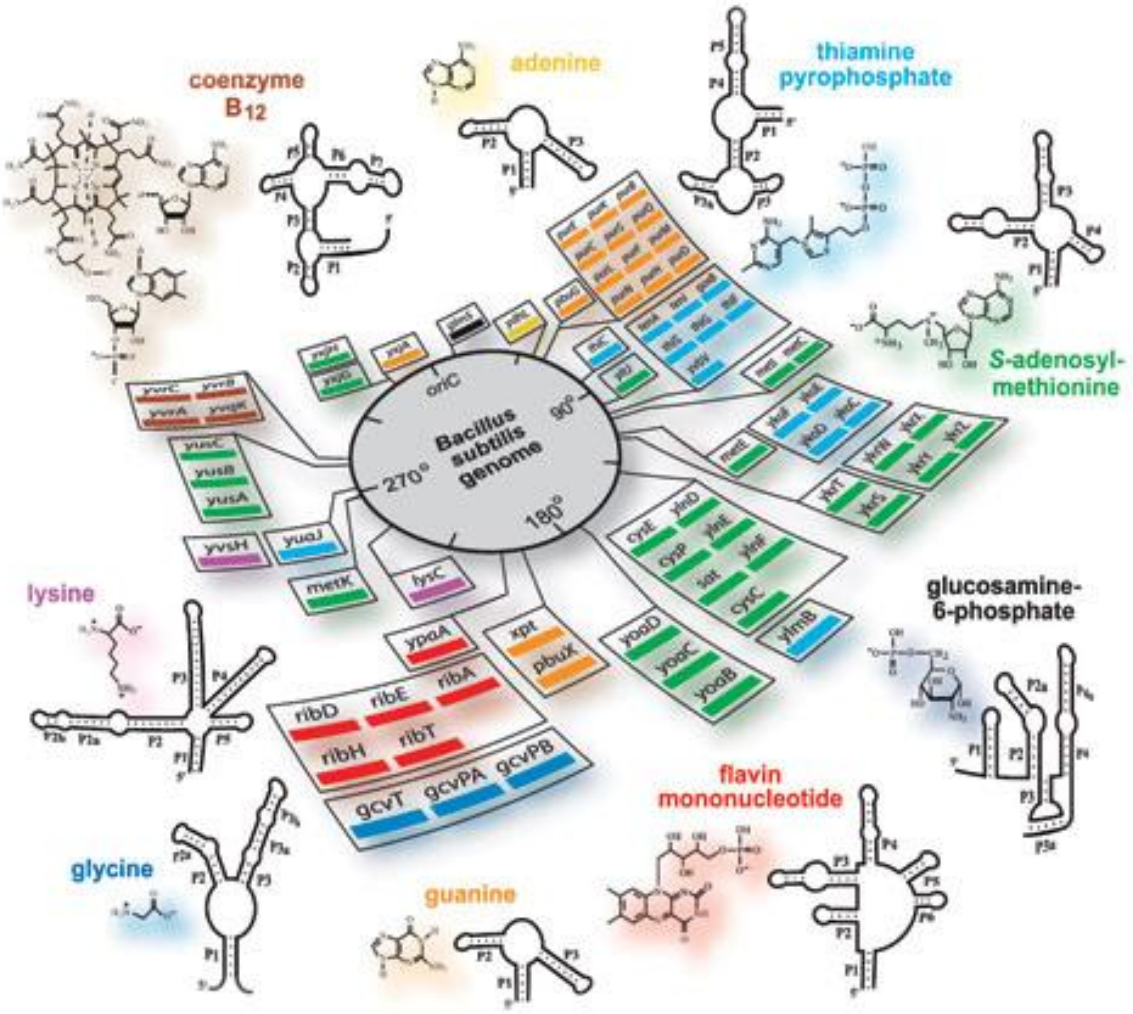


Figure 6. There are many classes of riboswitches. They are shown in the figure above.

Riboswitches function as sensors and are direct regulators of cellular metabolite concentrations because the genes regulated by them are generally involved in the synthesis or transport of their cognitive metabolites. They modify the expression of biosynthetic and transport proteins by binding to particular ligands. These ligands that bind to specific riboswitches are amino acids, nuclear bases, and sugars such as guanine, adenine, lysine, glycine, and thiamine pyrophosphate. The result of ligand binding is a structural change in the riboswitch, which affects the ability of RNA polymerase to continue the process of transcription elongation or the ability of the ribosome to translate an mRNA. The former affects gene expression through the formation of intrinsic transcription terminator structures. Although the transcription elongation structure of the RNA polymerase is stable, it is broken down by DNA termination sequences. The terminator stem-loop, which directs RNA polymerase, will stall the polymerase by acting as a roadblock (8). The RNA polymerase will simulate the release of the RNA which is done by rho-independent terminators (9) as can be seen in Figure 4. Translation switches regulate expression sequestration of the ribosome-binding site.

Riboswitches consist of two domains. One is the aptamer domain, which involves ligand binding. The other is the expression platform, which detects the changes in gene expression. Most riboswitches recognize single metabolites to modulate gene expression. If the metabolite is not present in the 5′ untranslated regions when it is transcribed, the riboswitch most times will refold into a structure that does not interfere with the expression of the open reading frame. However if

the metabolite is present in a high enough concentration when the 5′ untranslated regions is transcribed, it will induce a conformational change in structure, which will repress the expression of the open reading frame. As stated previously this structure will act as a terminator of transcription or sequester to translation, which prevents the ribosome from binding to the mRNA and translating the open reading frame.

New RNA Motifs Suggest an Expanded Scope for Riboswitches in Bacterial Genetic Control

Within mRNA there are highly structured domains that control gene expression and sense metabolites known as riboswitches. They have characteristics similar to protein genetic factors and transcription factors and modulate transcription or translation. “Riboswitches are versatile genetic control elements” that can account for why the same aptamer class in the same prokaryotic organism can control both transcription and translation (13). Also discovered is that mRNA processing can be used by riboswitches to modulate gene expression which led to the discovery of the first ribozyme riboswitch (13).

“Riboswitches form ligand-receptor interfaces with a level of structural complexity and selectivity that approaches that of proteins” and data provides “compelling evidence that riboswitches form structured receptors that are among the most selective of any RNA drug target” (12). This means that one can make highly selective riboswitch-targeting compounds that do not bind to other cellular molecules. When a metabolite binds to the riboswitch it induces a conformational

change of the mRNA. Due to the metabolite binding, the mRNA switches the conformation from one that is favorable to the expression of the

encoded proteins to one that is not favorable. RNA structures are complex and highly specific for one particular metabolite. RNAs discriminate between and do not allow binding of even very closely related metabolites, because of this these structures can be mutated so they can recognize new metabolite targets (14).

Metabolite binding can be a kinetically or thermodynamically directed process. When the rate of the metabolite binding to the RNA exceeds the rate in which the riboswitch is folding then the process is kinetic. On the other hand when the metabolite binding occurs and the riboswitch structure is more stabile compared to the other mRNA conformations, the process is thermodynamic (15). As well as RNA folding, the rate of transcription elongation by RNA polymerase plays a role in kinetic and thermodynamic control of riboswitches. The rate at which RNA polymerase synthesizes mRNA is dependent on the concentration of nucleotide triphosphates, Mg^{2+} ions, transcription factor, and the sequence of the DNA template. “A process that enables conformational rearrangements to occur, especially in the presence of Mg^{2+} ions that can influence the tertiary structure of an mRNA, could lead to more efficient ligand binding and hence, heightened gene expression control by the riboswitch” (15).

Organisms use RNA in many regulatory mechanisms to control gene expression. The primary examples are transcription and translation in bacteria. The idea of these mechanisms is that an outside source that generally has to do with the metabolic status of the cell, controls the formation of a stem-loop RNA structure that

will either terminate transcription too soon or sequester the Shine-Dalgarno (SD) sequence and prevent translation initiation (see Figure 7).

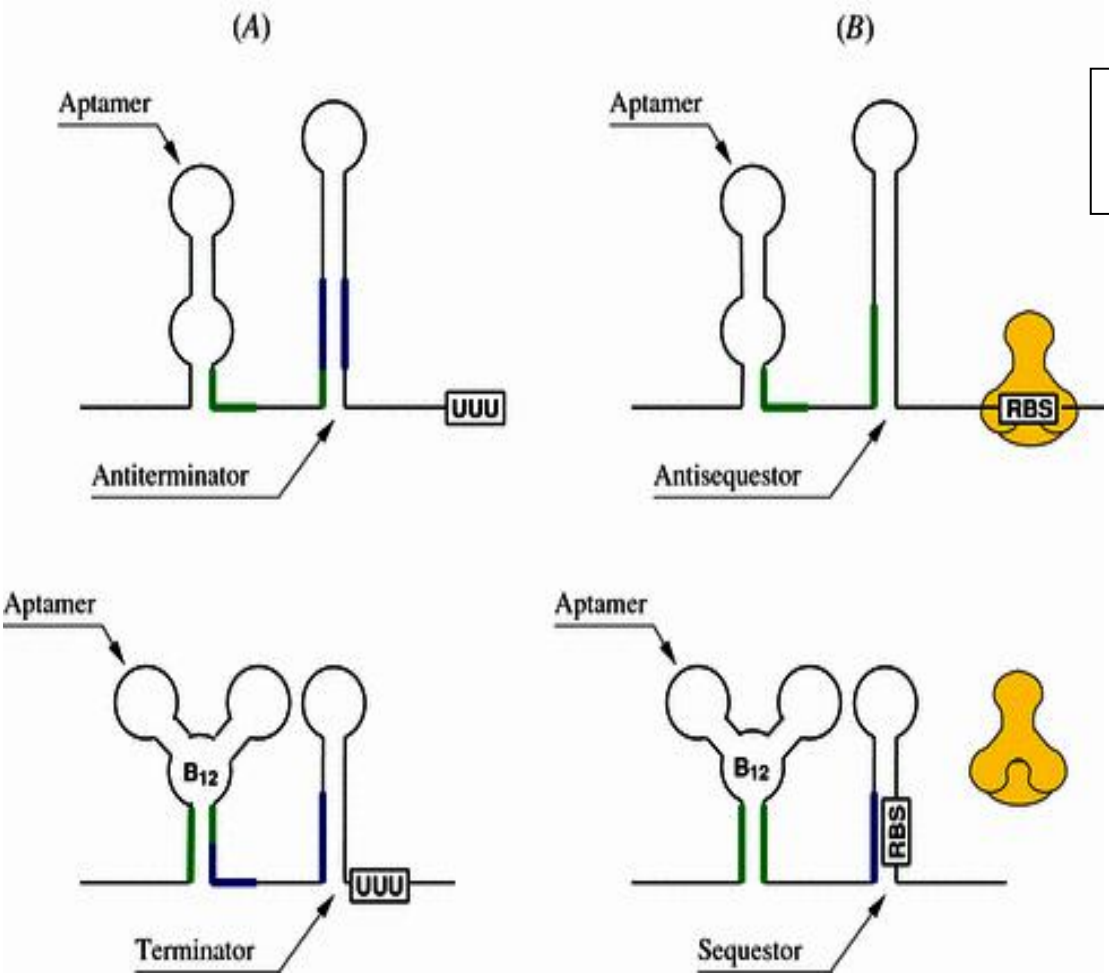


Figure 7 Showing the secondary structure of a riboswitch. It is indicating the aptamer and expression platform that will form a terminator, antiterminator, squestor, or antisequestor.

Protein factors have been implicated in the regulation of these processes. Some proteins control the formation of other RNA structures that act as an intrinsic transcription terminator or SD sequester, or even the antiterminator or

antisequester. In many cases the leader region that binds the factor can form a third structure. It can form the anti-antiterminator or the anti-antisequestor. These enable the protein to control transcription termination or translation initiation at a large distance from the terminator or ribosome-binding site. Bacterial riboswitches repress or activate their genes depending on the configuration of the corresponding leader RNA sequence. If there is a change in the metabolite concentration, the riboswitch can control transcription termination, translation initiation, or both if the stem-loop structure of the terminator also serves as a sequester of the ribosome-binding site. The riboswitch structure will be stabilized if a specific metabolite is bound to the conserved RNA-sensor domain. This will prevent an alternate RNA structure from forming which could be the terminator, sequester, antiterminator, or antisequester of the ribosome-binding site.

Gene Regulation by Riboswitches

The expression of many genes in a cell constantly needs to provide the correct levels of RNA and protein production. Gene-control systems must be able to do all the same things a normal cell would be able to carry out. Therefore they need to be very specific, rapid, and efficient in the level of expression of many genes.

Riboswitches control gene expression. Based on the leader RNA sequence bacterial riboswitches will activate or repress gene expression. Depending on how the metabolite concentration changes the riboswitch controls transcription termination or translation initiation, perhaps both if the stem-loop structure of the terminator is also a sequestor of the ribosome binding site. The riboswitch structure is stabilized when the specific metabolite is bound to the conserved RNA-sensor. This will prevent the alternative structure from forming which may be an antiterminator, antisequester, terminator, or sequestor (16). Intrinsic terminators are extended stem-loop structures that allow the RNA polymerase to stop transcription before the coding part of the mRNA is formed. Before the metabolite concentration reaches its threshold, the ligand will not bind to the aptamer domain. This will allow formation of an antiterminator stem, which will

allow complete transcription of the mRNA. However if the metabolite concentration threshold is reached, an allosteric change will occur producing the intrinsic terminator and preventing gene expression (17). (Offswitch). Likewise riboswitches may trigger transcription or translation by unfolding a terminator or unmasking the SD sequence (Onswitches).

(3) YkkCD Riboswitch

Efflux pumps are proteins that transport toxic substrates (including antibiotics) out of the cell and into the external environment. Pumps maybe specific to a single metabolite or can transport compounds that are different structurally. These are typically involved with multiple drug resistance (MDR). There are five classes of efflux pumps: major facilitator (MF), multidrug and toxic efflux (MATE), resistance-nodulation-division (RND), small multidrug resistance (SMR) and ATP binding cassette (ABC) (18). The ykkCD efflux pump is a part of the SMR family of efflux pumps (19). It is a heterodimer pump with broad ligand specification that includes streptomycin, ethidium bromide, etc.

The sensor that we are studying is the ykkCD riboswitch. This sensor can be found in the 5' untranslated region of the ykkCD efflux pump gene. This sensor is specific to tetracycline and only recognizes the antibiotic tetracycline, but the aptamer domain binds to minocycline and anhydrotetracycline as well. The level of gene expression can be affected at the transcription or translation stage of protein production. When the antibiotic concentration reaches its threshold an allosteric

conformational change is induced which at the transcription stage unfolds the terminator stem. On the other hand at the translational stage the ribosome binding site will be unmasked. When the Bacillus subtilus cells were grown with tetracycline, there was an increase in the levels of efflux pump mRNA, which indicates regulation at the transcription stage. Runoff transcription assays in the presence of tetracycline were done that indicated the riboswitch sensor is sufficient to increase the level of the pump mRNA (Figures 8 & 9). These assays were performed in vitro containing only the riboswitch and the efflux pump to verify the results. When the riboswitch is off, only the riboswitch is produced because the terminator stem stops the polymerase. However when the riboswitch is on, an allosteric conformational change occurs where both the riboswitch and the pump are produced. This will produce a longer transcript.

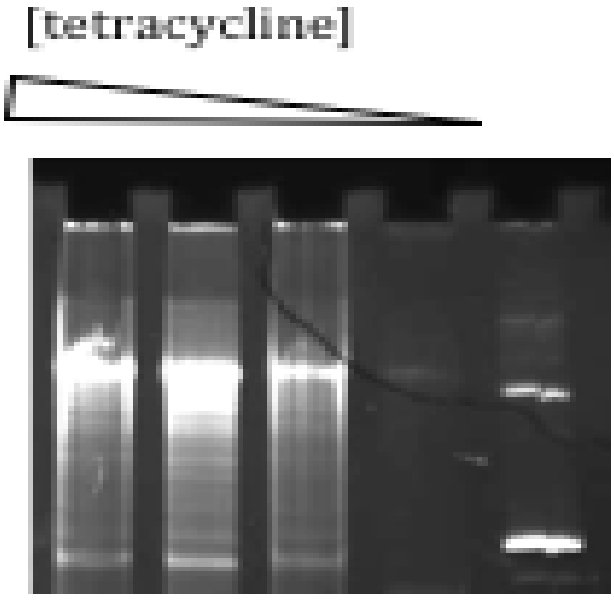


Figure 8. Runoff transcription is shown here.

Figure 9. The ykkCD riboswitch and tetracycline appears sufficient to increase pump mRNA levels thus upregulating expression if the ykkCD efflux pump.

(4) The YkkCD Riboswitch sensor

The riboswitch sensor has two parts. The first part is the aptamer domain, which specifically recognizes tetracycline. The second part is the expression platform that undergoes an allosteric conformational change to regulate the level of pump production. Also at work is the terminator stem, which acts as a roadblock for the polymerase. It does this by stopping the polymerase, which falls off the DNA and does not make any more RNA. (See Figure 10).

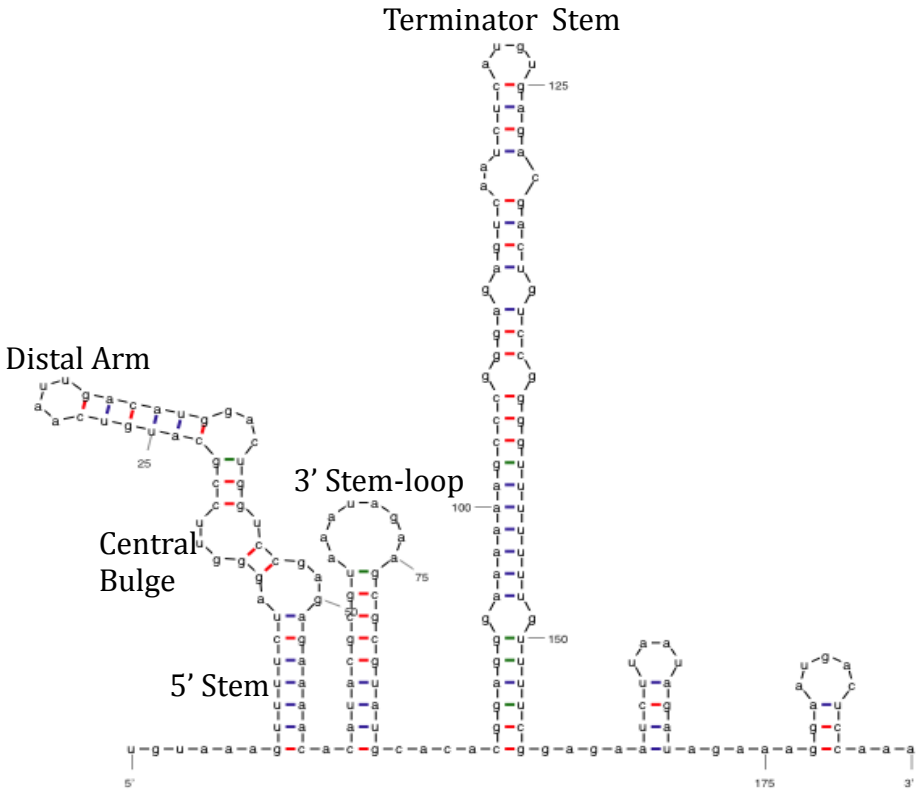


Figure 10. The predicted secondary structure of the ykkCD riboswitch. On the left is the aptamer and on the right is the expression platform.

(a) Lock and Key Model

RNA aptamers of riboswitches are considered complex structures that are able to specifically recognize their target molecule rejecting any closely related compound. Their specificity rivals that of protein receptors. How is RNA capable of achieving such specificity? Thus we need to uncover the interaction between tetracycline and the sensor RNA must be determined to understand how it

specifically recognizes the antibiotic. The molecular basis of specific recognition can be understood using a simple lock and key model (See Figure 11). The antibiotic would be analogous to the key and the riboswitch to the lock. Only a perfect fit between the sensor and the antibiotic will trigger production of the resistance gene. Next, the functional groups which are important in tetracycline for recognition and which part of the riboswitch sensor is important for recognition must be determined.

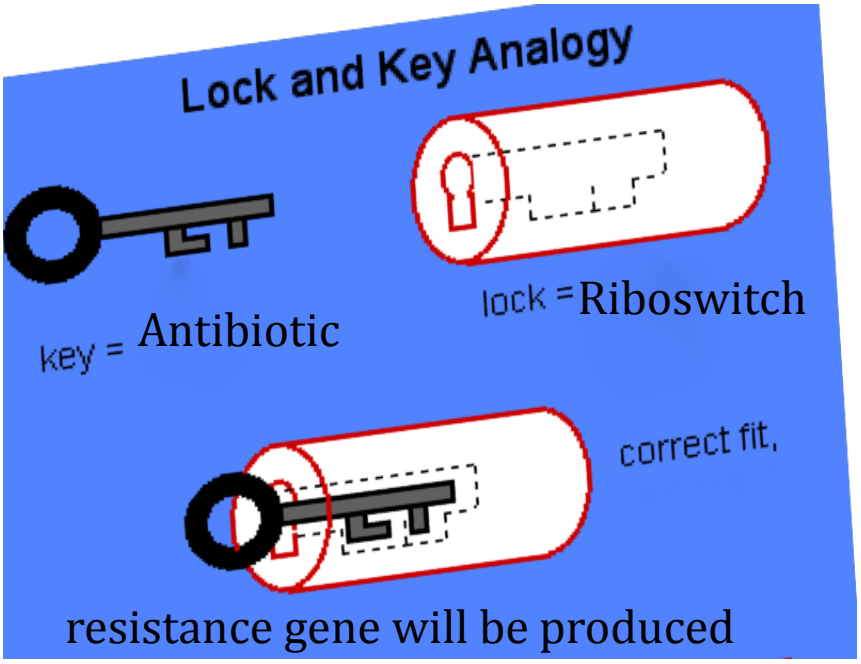


Figure 11. The lock and key model is an analogy used to simplify how the ykkCD sensor specifically recognizes tetracycline.

To evaluate the tetracycline-sensor RNA binding affinity, a high-throughput assay had to be developed. The idea behind the assay is an energy transfer between

two chromophores. When a fluorescing chromophore absorbs light, the energy can be released as light in a process known as fluorescence. If another chromophore is within about 100 amino acids of the first and absorbs the light at the wavelength of emission, the energy will likely be transferred. This is known as quenching (20) see Figure 12. The amount of chromophore in close enough range to quench the other is dependent on the amount of ligand present in the assay medium. The materials are placed in an aqueous medium that contains buffer, incubated, and irradiated with light absorbed by the molecule undergoing fluorescence. (20). The amount of ligand can be determined once a time interval is determined or the system has reached equilibrium, and the results are compared with a known standard. This system appears to be quick and easy; however, there are many problems that can occur and many pieces to resolve before the assay can be completed. The RNA has to be made and it has to be in its native-like conformation. The RNA needs to be pure when adding it to the assay, so that there is very little of anything else affecting the results. When the RNA is purified it is denatured and thus has to be refolded to reach its native-like structure.

Mg^{2+} plays a significant part in the structure and function of many RNAs. It has been shown to stabilize the tertiary structure of RNA under conditions that only weakly affect the stability of the secondary structure. Mg^{2+} levels when increased enable tighter metabolite binding. This will lead to a reorganization of the binding pocket in certain riboswitches. Thus it is important to optimize the appropriate

magnesium concentration and RNA refolding procedure before binding assays are conducted.

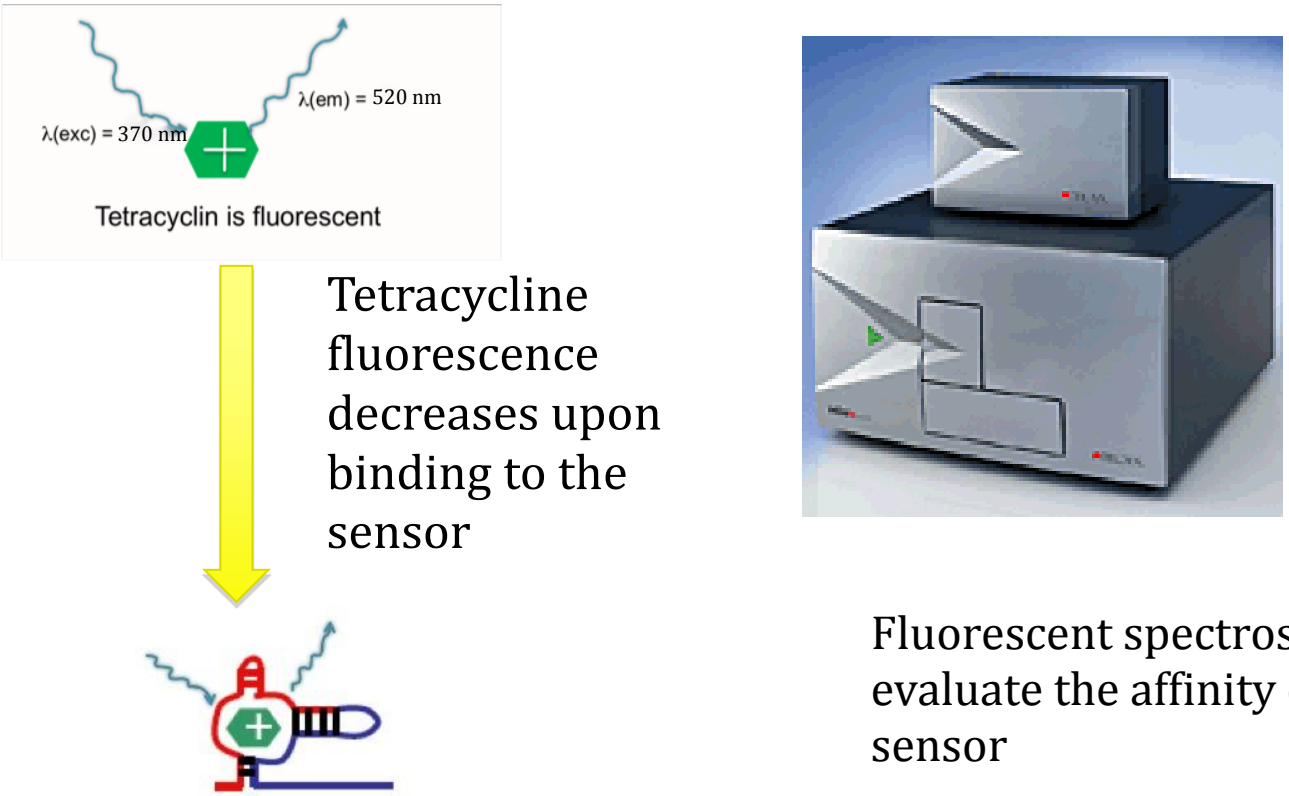


Figure 12. In the middle the infinite 200 plate reader to read out the fluorescent quenching assays. To the left is a schematic of fluorescent quenching and to the right is an example result.

(b) Tetracycline and Riboswitch Sensor Recognition

To test the importance of tetracycline functional groups we tested four tetracycline derivatives and tetracycline. The derivatives were anhydrotetracycline,

doxycycline, minocycline, and oxytetracycline. Figure 13 shows how they differ structurally from tetracycline, the base antibiotic.

Because tetracycline and its derivatives are fluorescent, the same high-throughput fluorescent quenching assay can be used to find their binding affinities (K_D value). Specifically due to the excitation and emission wavelengths being similar and very close in range as can be seen in Table 1, it is possible to use the same assays by adjusting the wavelengths on the fluorescent plate reader. This is helpful because it is quick and less material is required

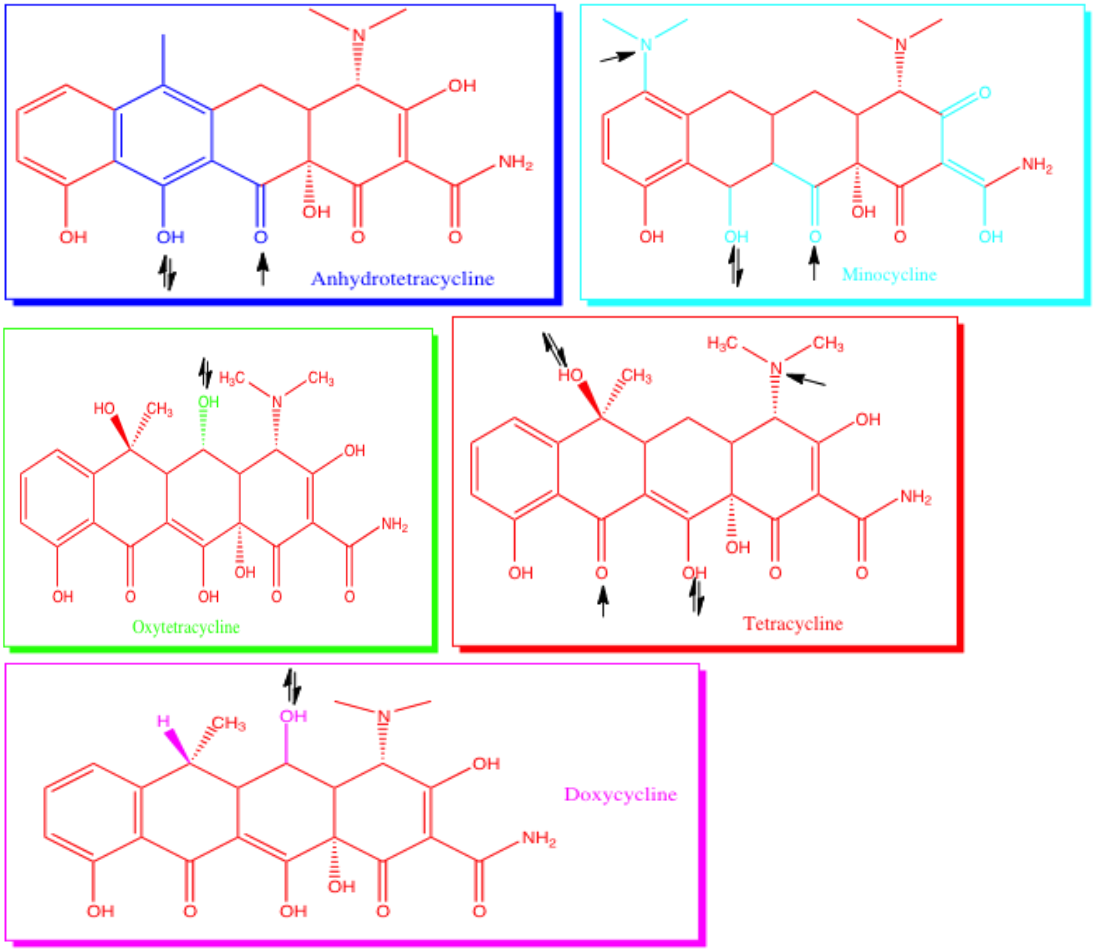


Figure 13. Tetracycline and derivatives structure. Colors indicate how each structure is different from tetracycline. Arrows indicate if a functional group acts as H-bond donor, acceptor or both.

Table 1.

	Excitation	Emission
Tetracycline	370 nm	520 nm
Anhydrotetracycline	440 nm	545 nm
Doxycycline	430 nm	510 nm
Minocycline	450 nm	480 nm
Oxytetracycline	430 nm	530 nm

To test which part of the riboswitch is important for recognition, site directed mutagenesis of evolutionary conserved riboswitch regions was performed. The nucleotides conserved through evolution are believed to be important for recognition. By using multiple sequence alignment of sensor sequences from 15 different bacterial species, the conserved nucleotides were identified. These invariable blocks were subjected to site-directed mutagenesis. The ability of these mutant sensors to recognize tetracycline was evaluated using binding assays. If the mutated sensor lost the ability to recognize tetracycline, the invariable block is important for tetracycline recognition. Figure 14 shows the multiple sequence alignment for 15 gram (+) bacterial species. The red areas show the invariable blocks conserved more than 90%. The blue areas show the invariable blocks conserved more than 50%. The invariable blocks marked in red which are located

on the secondary structure of the riboswitch sensor seen in Figure 14 has the highest conservation and is likely to be involved in tetracycline recognition.

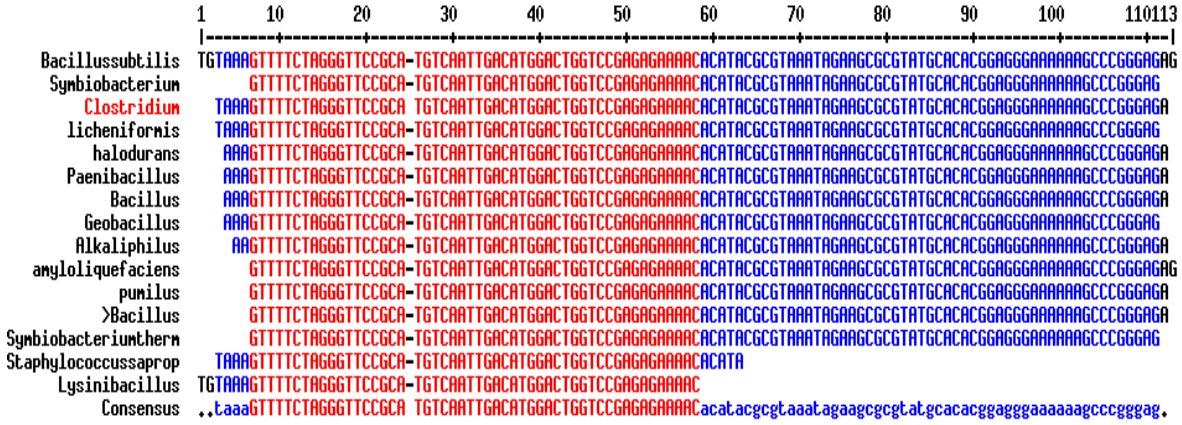


Figure 14. The invariable blocks in red are 90% conserved and are most likely involved in tetracycline recognition.

(c) Quikchange Site-directed Mutagenesis

Quikchange site-directed mutagenesis is used to make the ykkCD sensor mutants. For this procedure, PCR is the first step. The circular plasmid DNA that contains the wild type DNA sensor is analyzed to find one spot which is indicated by a bullet circle in Figure 15 the wild type DNA will be changed to the new mutated DNA. The process requires primers, which are designed to change the sequence (short DNA sequence) with the mutated sequence desired. The polymerase then synthesizes the plasmid DNA containing the mutated DNA sequence. Once the sequence is made, the

wild type DNA needs to be removed. This is accomplished by adding the DpnI enzyme. The wild type DNA is methylated and the enzyme digests the methylated wild type DNA. A transformation is then required to produce more of the DNA sequence wanted (Figure 15).

1. PCR Procedure

To perform the PCR there are many steps to complete.To a PCR tube, 25μL of Pfu master mix buffer, 1 μL of the top primer, 1 μL of the bottom primer. Add 1 μL of the wild type DNA, 23 μL of RNase free water were added. All liquids were centrifuged before being placed into the PCR machine, using the program setting 465 lab, which has the correct time intervals for its respective temperature, i.e. 95° C for 1 minute. After the DNA has been made, 1 μL of DpN1 enzyme was added to digest the wild type DNA. Once the wild type DNA has been digested the transformation can be completed.

2. Transformation Procedure

10μL of the mutated DNA was added to 100 μL of the Dh5α competent cells. The cells were stored on ice for 20 minutes. The cells were then heat shocked for 2 minutes at 42° C. The cells were cooled on ice for 2 minutes. The cells were incubated and grown in a shaker at 37° C, 120 rpm for one hour. 900 μL LB media was added to labeled sterile glass tubes. The 110 μL of cells were transferred to the media in the glass tubes. After the cells were grown, the cells were transferred to

sterile centrifuge tubes. The cells in solution were spun for 2 minutes to extract the cells from the media. 900 μL of LB media was removed and the cells were resuspended in the remaining 100 μL of LB media. The cells were plated on a LB agar and 150 μg/mL ampicillin plates (Sigma, #A0166-25G). The plate was placed into a 37° C incubator overnight for about 19 hours. Once the colonies were grown, they were inoculated in 20-40 mL of LB broth and grown overnight for about 19 hours.

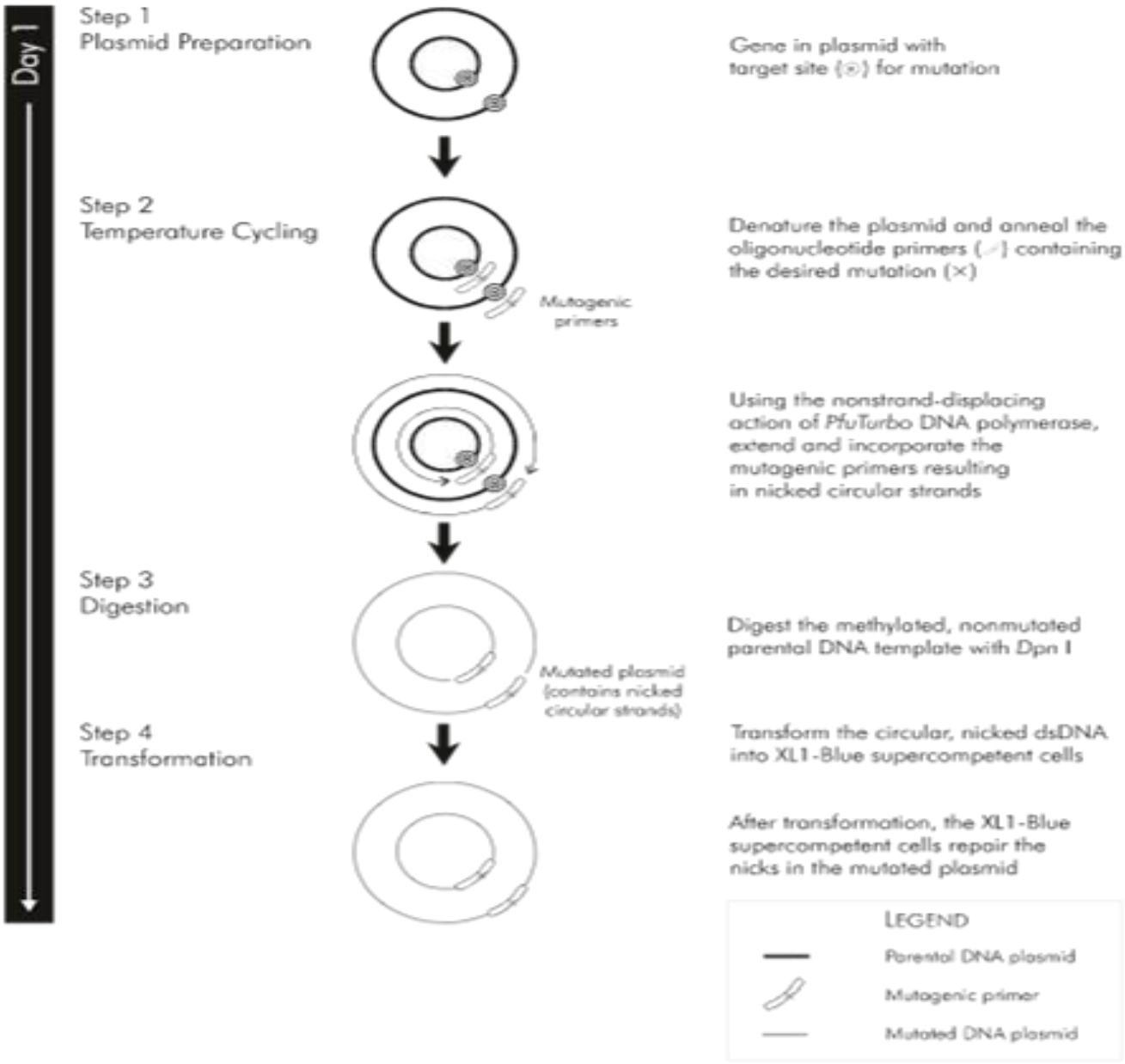


FIGURE 1 Overview of the QuikChange® site-directed mutagenesis method.

Figure 15. Quickchange PCR mechanism.

(e) Plasmid Prep

Next, the plasmid prep was performed. In the procedure the mutant sensor DNA will be purified from the bacterial cells. The quality of plasmid DNA can be evaluated using an UV spectrophotometer and agarose gel electrophoresis. A traditional plasmid prep was begun with an overnight inoculation of harvest cells grown by centrifugation. The bacterial culture was spun down leaving only the cells. Next the lysis procedure was performed. Lysozyme solution (which degrades the cell wall), alkali (which punches holes in the cell membrane), and the neutralization solution were added. Next the extract was spun. The extract was cleaned with phenol and chloroform (or by column purification). The DNA was precipitated with alcohol. The DNA was concentrated; cells were spun to pellets and resuspended in sterile water or TE.

1. **Five Minute Plasmid Miniprep Procedure**

   A Sigma-Aldrich GenElute Five-Minute Plasmid Miniprep kit (Sigma, PFM 10, PFM 50, PFM 250) was used for DNA isolation according to the manufacture's instructions. The culture was lysed. 40 μL of constituted lysis reagent was added to 400 μL of overnight culture in a 2 mL collection tube. It was mixed by rapid inversion and incubated for 2 minutes. The binding column was prepared. 500 μL of column prep solution was added to a binding column seated in a 2 mL collection tube. The solution was spun for

10 seconds and the flow through was discarded. The DNA was bound to the column. 400 µL of binding solution was added to the tube and mixed by inversion 15 times. The roughly 780 µL of mixture was added to a pre-washed binding column and spun for 20 seconds. The flow through was discarded. 700 µL of diluted wash solution was added to the column and spun for 20 seconds to remove impurities. The flow through was discarded. 200 µL of diluted wash solution was added to the column and spun for 30 seconds. The plasmid DNA was eluted from the column. The column was carefully transferred to a new 2 mL collection tube, 40 µL of elution solution was added, and the column was spun for 30 seconds. For larger volumes, the large volume plasmid prep was used.

2. GenElute Plasmid Midiprep Kit

A Sigma-Aldrich GenElute Plasmid Midiprep kit (Sigma, PLD 35, PLD140) was used for DNA isolation of larger volumes according to the manufacture's instructions. The cells were harvested overnight. 5-40 mL of overnight recombinant E. coli culture were spun to a pellet by centrifugation. The appropriate volume of recombinant E. coli culture was added to an Oak Ridge style tube. The solution was centrifuged at 3,000-5,000 xg for 5-10 minutes. The entire medium supernatant was removed and discarded. Next the cells were resuspended in solution. The bacterial pellet was resuspended with 1.2 mL of resuspention solution by pipetting up and down. Then

similarly in the miniprep, the cells were lysed. Adding 1.2 mL of the lysis solution lysed the resuspended cells. The solution was mixed immediately by gentle inversion 6-8 times until the solution becomes clear. Do not exceed 5 minutes. Next the solution was neutralized. 1.6 mL of the neutralization/ binding solution was added to precipitate the cell debris. The tube was gently inverted 4-6 times. The cell debris was spun to a pellet by centrifuging at or above 15,000 xg for 10-15minutes.

(f) Agarose Gel Electrophoresis

An agarose gel is a technique, which separates DNA by molecular weight. However in the process it does not denature the DNA. Since the gel does not denature the DNA, molecules migrate based on their charge and their shape. The supercoiled and circular DNA can be distinguished in this method. Also because the DNA is negatively charged primarily due to the phosphodiester bonds, there is no need to coat the DNA with a molecule that is negatively charged. This is important because the molecules migrate in an electric field from negative to positive. In order to estimate the size of the DNA based on comparison, a molecular weight marker is used. To see the DNA, the agarose gel is stained with ethidium bromide (EtBr). The EtBr fluorescent molecule chelates with DNA bases so that is can be observed. The EtBr that is not chelated with the DNA migrates out of the gel quickly due to its small size. The DNA then fluoresces under UV light because the EtBr is fluorescent. To run the DNA samples, 10 μL of the DNA with 2 μL of 6x loading dye was loaded

into the wells of the gel. The machine was set to 100 V and run for at least 20 minutes. The gel was exposed to UV light to take a picture.

1. **Preparing Agarose Gel**

   A cork Erlenmeyer flask is used. 1 gram of agarose was added to the flask. Then 100 mL of 1x tris-acetate-EDTA (TAE) buffer was added. The flask and contents were microwaved for one minute or until it was boiling. Running cool water on the outside of the flask cooled the flask. 10 µL of EtBr was added for staining. The contents were poured into the gel plate. It sat for 15 minutes to set.

(5) Preparing DNA for RNA synthesis

(a) Linearization of the DNA Protocol

For a 100 µL reaction mixture, = 10 µL of XbaI or BamHI restriction enzymes was added. 10 µL of 10x buffer 4 was added for XbaI or buffer 3 for BamHI. Then 10 µL of 10x BSA was added to the mixture. 70-75 µL of the DNA was added for linearization. The mixture was placed in the water bath for 1 hour. This process was optimized as we found that an hour was not long enough for the process to be completed and the maximum amount of enzyme was necessary. At first the time was increased to 3 hours, but that was not a long enough time. The overnight in the water bath and the maximum 10% of enzyme was found to be optimal for the linearization (Figure 16).

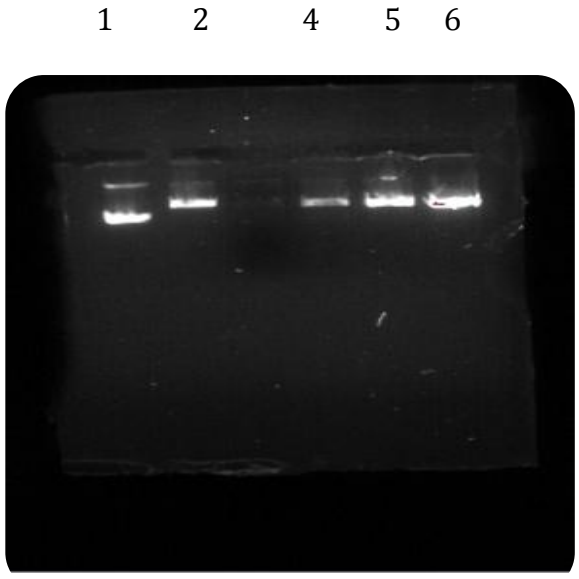


Figure 16. Linearization of Y DNA on an agarose gel. Lane 1 represents supercoiled (uncut) DNA; Lanes 2,4,5,6 represent linearized DNA.

(b) Phenol Chloroform Extraction Procedure

For a 100 µL reaction, 100 µL of phenol chloroform (25 phenol: 24 chloroform: 1 isoamyl alcohol) was added but we made sure to take the bottom layer of the mixture. The tube was vortexed for 20 seconds. The tube was spun for 2 minutes to separate the layers. The bottom layer will be the layer of phenol chloroform, which was removed. Next 100 µL of chloroform was added to the tube and vortexed for 20 seconds to mix. Once again the tube was spun for 2 minutes to separate the chloroform from the desired DNA. The bottom layer was removed. Finally 10 µL of sodium acetate and 100 µL of isopropanol were added to the mixture. The tube was vortexed for 20 seconds as previously done. The tube was placed in the -20° C freezer for precipitation.

(c) Plasmid Prep Precipitation Procedure

The plasmid DNA was spun for 30 minutes at maximum speed of ~13,500 rpm at 4° C. The supernatant was carefully removed. 50 µL of cold 75% ethanol was added. The plasmid DNA was spun at maximum speed for 5 minutes at 4° C. The supernatant was removed. The DNA pellet was dried using a speed vacuum for 5 minutes. The pellet was resuspended in 50µL of 1x tris-HCl and EDTA (TE). The concentration of the plasmid DNA was checked by UV spectrophotometer. An agarose gel was run and a picture was taken.

(d) Transcription: DNA to RNA Procedure

The transcription has been done a few different ways. Each way appears to work about the same. The reason for using different methods is optimization and availability of materials. The first recipe for a 50µL transcription, 1µL of T7RNA enzyme, 12 µL of DNA as long as it adds up to be 1µg, 5µL of 10x buffer, 5µL of rNTP, which needs to be 0.5 µM in each reaction, and 27 µL of RNase free water were added. The reaction mixture then went in a water bath for 1 hour and 30minutes at 37° C. After the transcription was complete, a urea gel was run to check that the transcription was complete.

The second method for a 50 µL reaction was done. 1 µL of 1M Tris, 5µL of 10% T7RNA enzyme, varying volume of DNA as long as it adds up to be 1µg, 2.5µL of 100mM DTT, 12µL of 100mM $MgCl_2$, 0.5 µL of 1% Triton, 2.5µL of rNTP, and varying

volume of RNase free water depending on the amount of DNA were added. The reaction mixture sat in the 37° C water bath for 1 hour and 30 minutes just as the previous method. Because the materials for this method are more readily accessible, it had become the dominant method for transcription.

The final method of transcription for a 20 µL reaction was done. 4 µL of linearized DNA, 10 µL of 2x buffer, 2 µL of enzyme mix, 1 µL of RNase inhibitor, and 3 µL of RNase free water were added. The mixture was placed in to the 37° C water bath for 30 minutes. This was the most efficient method however the least available (Figure 17).

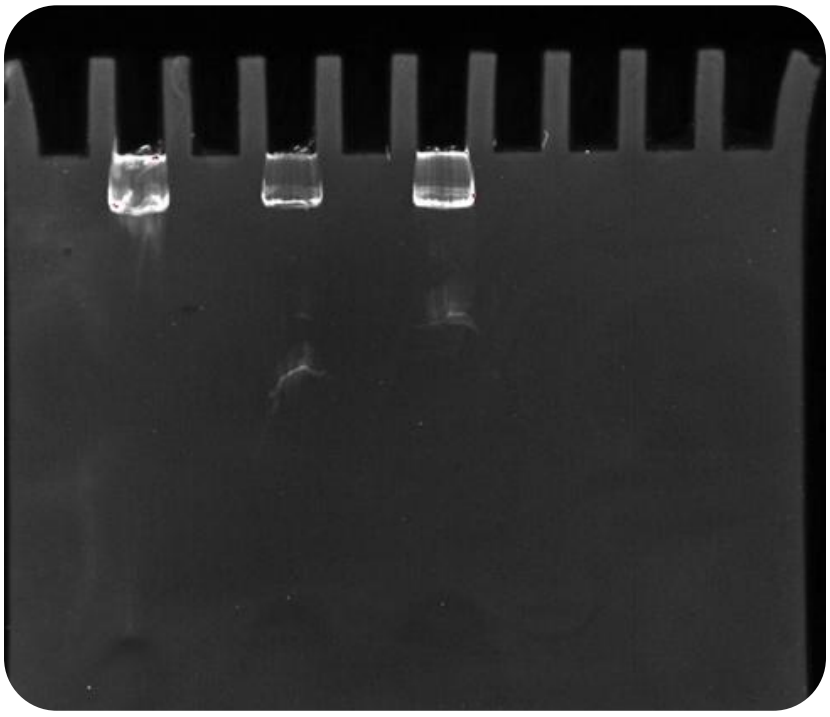


Figure 17. Transcription of Y DNA to RNA on a small urea gel.

(e) RNA Clean Up

Once the RNA has been made, it must be cleaned. In other words the RNA must be purified from salt, supercoiled and unsupercoiled DNA, polymerase, buffer, and RNA degradation products. These may interfere with the binding assays. There are many ways to clean the RNA. Multiple methods and kits have been tried to find the best results, which will be explained.

Methods:

(a) RNA Cleanup Kit 1

RNeasy Mini kit (Qiagen, 74104) was used according to the manufacturer's instructions. First the sample was adjusted to a volume of 100 µL with RNase free water. 350 µL of RLT buffer was added and mixed well. Then 250 µL of pure (96-100%) ethanol was added to the diluted RNA and mixed well by pipetting. The sample was transferred to an RNeasy Mini Spin column and placed in a 2mL collection tube. The sample was centrifuged 15 seconds at 8,000 xg (≥ 10,000 rpm). That flow-through was discarded. 500 µL of buffer AE was added to the RNeasy spin column. The mixture was centrifuged for 15 seconds at ≥ 10,000 rpm to wash the spin column membrane. The spin column was removed so it did not touch the flow-through. Next the RNeasy spin column was placed in a new 2mL collection tube and the old collection tube was discarded. The solution was centrifuged at full speed for 1 minute. The RNeasy spin column was placed in a new 1.5 mL collection tube. 30-50

µL RNase fee water was added directly to the spin column membrane. Last the column was centrifuged for 1 minute at ≥ 10,000 rpm to elute the RNA.

(b) RNA Cleanup/ QIAquick Nucleotide Removal Kit

QIAquick Nucleotide Removal kit (Qiagen, 28304) was used according to the manufacturer's instructions. First 5 times the volume of buffer PN was added to the sample. The QIAquick spin column was placed in the provided 2 mL collection tube. The DNA was bound to the column. The sample was added to the column and centrifuged for 1 minute at 6,000 rpm. Then the flow-through was discarded. 750 µL of buffer PE was added and centrifuged for 1 minute at 6,000 rpm. The flow-through was discarded. The sample was centrifuged again for 1 minute at 13,000 rpm to ensure that the PE buffer was gone. The column was placed in a clean 1.5 mL collection tube and centrifuged for 1 minute. To elute the DNA, 100 µL of buffer EB was added and centrifuged for 1 minute at 13,000 rpm.

(c) Gel Purification

Gel purification was the last method and most current method used to clean our RNA. It was the most consistent method with the best results. However it was also the most time consuming method. To complete this purification first a large urea gel was made with larger wells to load the sample. The gel was run for 30 minutes with the watts of minimum 35W to

heat the gel. While the gel was running, the RNA sample was heated for 10-15 minutes at 100° C to refold the RNA. The sample was spun for a short time. After the gel was done running, the wells were cleaned and the sample was loaded. The sample did run through the gel for 3 hours. This was done so the sample separated, in particular the RNA separated from the DNA, salt, and other impurities. Next a UV lamp was used to find the RNA band and the band was cut out. Sterilized tweezers were used to place the gel bits into a sterile syringe. The gel pieces were crushed through the syringe into a sterile tube with 1-4 ml of RNase free water. The RNA was extracted from the gel bits. The gel bits were spun down and the aqueous layer was removed into fresh sterilized tubes. The liquid was froze and then vacuumed down to 100-200 μL. Finally the sample was froze at -80° C or used as needed.

Results:

(a) This method resulted in very low RNA concentration. This was most likely due to the molecular weight cutoff of the column. The molecular weight cutoff of the column is 130 nucleotides and the RNA in this study is 112 nucleotides long. As a result the RNA didn't bind well to the column and was likely lost in the flow-through.

(b) This kit is designed to remove unincorporated nucleotides and the polymerase from the transcription mixture. As a result it does not

remove the DNA template or the RNA degradation products. Thus it is unable to provide clean enough RNA for the assays.

(c) As stated previously the gel purification method is the newest method being used. It is a relatively inexpensive way to clean the RNA, with readily available material that can be made when needed. It is the most efficient method, producing the purest RNA of the three methods tried. The method is time consuming but gives the best results. The RNA separates well from the DNA, salt, and other impurities that may have come from the transcription.

Fluorescent Quenching (Binding) Assays

The idea behind these assays is to find the binding affinity between the antibiotic and the riboswitch. It helps to determine which region or regions of the riboswitch are important and which functional group or groups are important for the antibiotic. There are three possible outcomes of the mutagenesis studies done by this method. The mutant can improve the recognition, which will be indicated by K_D^{mutant}: $K_D^{wild\ type} < 10$. The mutant can destroy recognition, K_D^{mutant}: $K_D^{wild\ type} > 10$. Lastly, the mutant does not affect antibiotic recognition; K_D^{mutant} is within an order of magnitude of $K_D^{wild\ type}$. The first two outcomes will likely hit the sensor on spots that are important for recognition.

Method

First all necessary buffers were made. To make the 5x buffer: 1 mL of 1M Tris, 2.5 mL 2M KCl, 0.5 mL 100 mM $MgCl_2$ 6 mL RNase free water were added. To make 10 nM tetracycline, 0.004445g of solid tetracycline was weighed and dissolved in 10 mL of DMSO. From this point a serial of dilutions were done until 10 nM concentrations is reached. After the buffers and antibiotic mixtures were made the

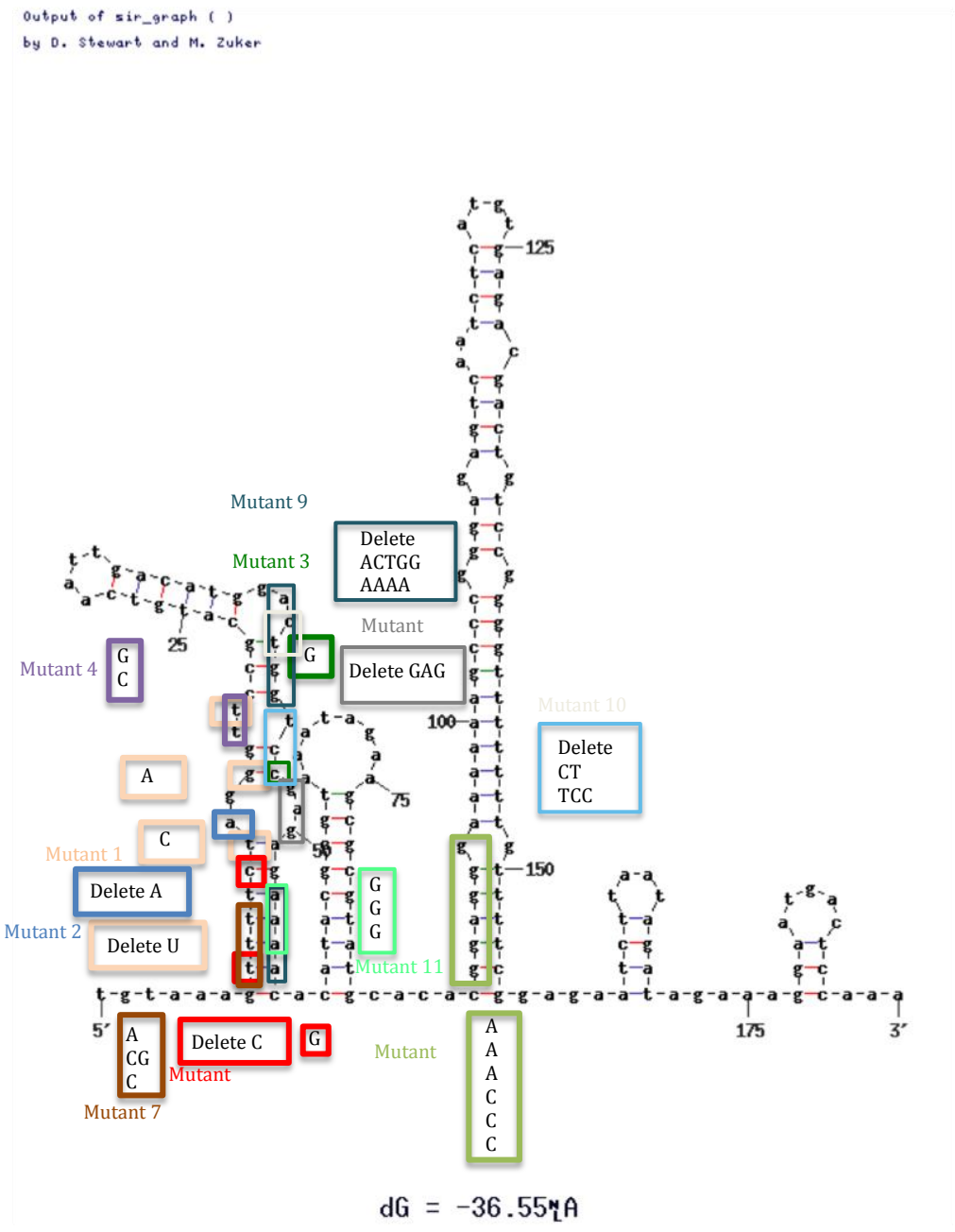
two solutions needed for the assays were made. One solution that contains RNA and one solution, which do not were made. For the RNA containing solutions, 10 µL of the 5x buffer, 5 µL of the tetracycline or derivative mixture, 25-35 µL of RNA, 1 µL of RNase inhibitor, and the rest is RNase free water were added to obtain a 50 µL solution. For the no RNA containing solution, 130 µL of the 5x reaction buffer, 65 µL of the 10 nM tetracycline or derivative mixture, and 455 µL of RNase free water were added together. Once all appropriate mixtures were made, 50 µL of the no RNA containing solution was added to each well. 50 µL of the RNA containing solution was taken and resuspended in the first well. 50 µL of that solution was added and resuspended in well 2; this was continued until well 11 and the last 50 µL of solution was thrown out. Finally the plate was covered with parafilm, covered with foil, and incubated for a minimum of 3 days to allow the reaction to occur.

The binding assays were read out. Use the I-control 1.6 program to control the infinite 200 plate reader and measure fluorescence of tetracycline and the derivatives. The appropriate excitation and emission wavelength, integration time, and number of flashes were chosen. The appropriate concentrations and fluorescent values were calculated and graphed using Graph Pad Prism. The K_D values were recorded.

Results

The K_D values of the eleven original mutants, four new mutants, tetracycline, and four commercially available derivatives were reviewed (Figure 18). The more

interesting results were any of those that changed the K_D value more than one order of magnitude within a 20% error (see Table 2). An order of magnitude change in K_D corresponds to about 1 kcal/mol in binding energy – roughly the energy of a H-bond. Any less of a change cannot be easily interpreted at the molecular level. According to Krystal Roark, there were three mutations in the original set of mutants, which were made on the central bulge in the secondary structure that cause the largest increase in magnitude of the K_D value. These were mutants 4, 8, and 10. Similarly in the second set of mutants which were tested, mutant 2 found in the same area became nonbinding (Figure 19). While mutant 7 was not in the same area it is located on the terminator stem of the expression platform, and it also did not bind. This particular area of the secondary structure has not been explored much, but also may be important. If this region of the RNA is altered it may cause the riboswitch structure to fold into an alternate structure that is not competent to tetracycline binding. These areas are believed to be important for tetracycline recognition and when changed or deleted likely cause a different structure formation that does not recognize tetracycline.


Output of sir_graph ( )
by D. Stewart and M. Zuker
Mutant 9
Mutant 3
Delete
ACTGG
AAAA
Mutant
Mutant 4
G
C
G
Delete GAG
Mutant 10
Delete
CT
TCC
A
C
Mutant 1
Delete A
Mutant 2
Delete U
G
G
G
Mutant 11
5′
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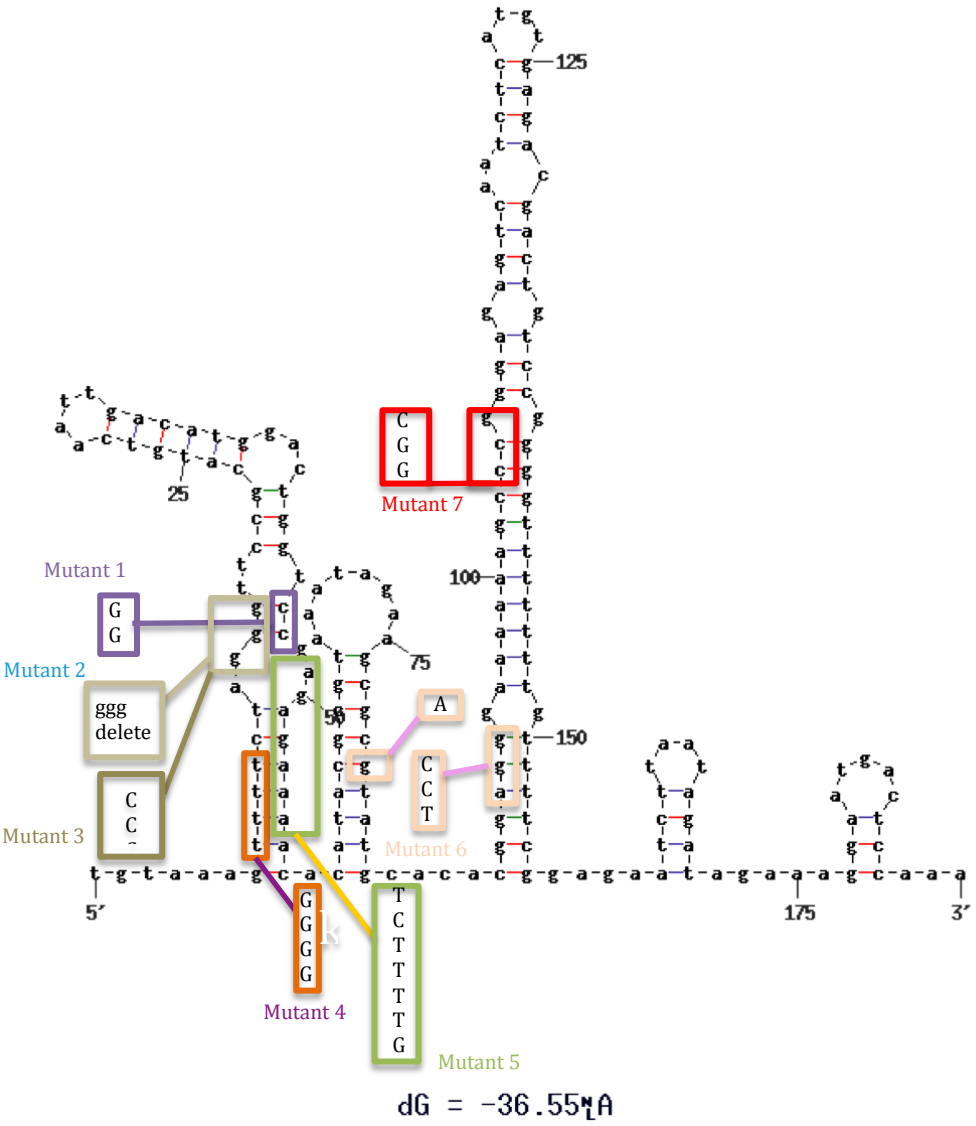
C
C
3′
dG = -36.55kA



My Mutants

Figure 18. Binding mutants mapped to the secondary structure of the riboswitch aptamer domain.

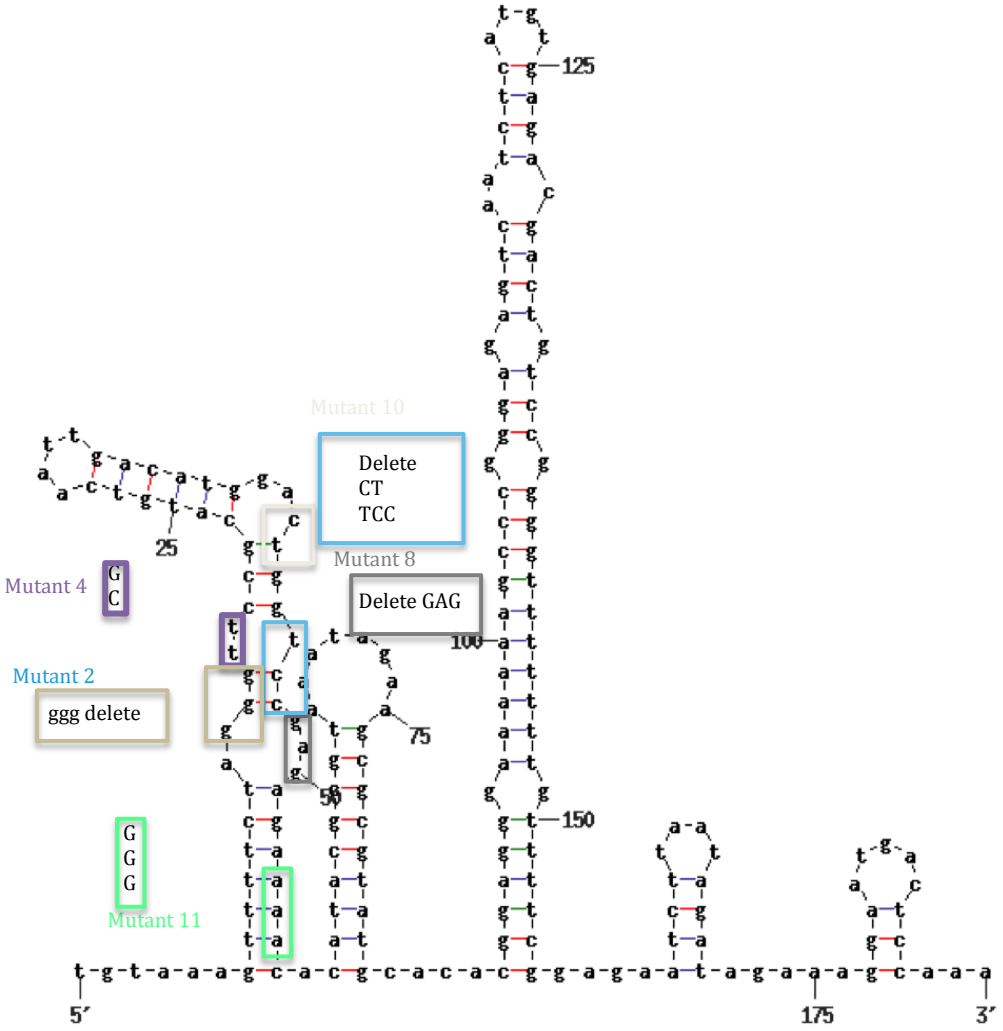


Figure 19. Nonbinding mutants mapped to the secondary structure of the ykkCD riboswitch. Nonbinding mutants appear to cluster on the central bulge of the riboswitch indicating a site for tetracycline binding.

Because it is known that the riboswitch sensor does recognize tetracycline, which functional group(s) of tetracycline are important can be determined by testing tetracycline derivatives. Several binding assays were performed, which gave

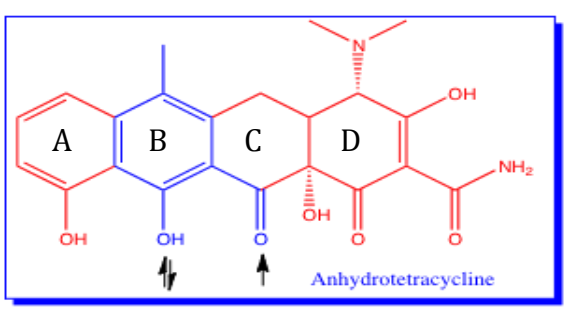
some unexpected results. In addition to tetracycline, minocycline, and possibly anhydrotetracycline, bind to the riboswitch. Minocycline when bound to the riboswitch caused a 10-fold increase in the K_D value. Anhydrotetracycline appears to bind, however more binding assays must be completed to be sure (Figure 20). From these results the lower side of the tetracycline structure is not as important to recognition, because minocycline changes that area significantly and binding was still retained albeit weakened. Both anhydrotetracycline and minocycline switches the carbonyl and hydroxyl groups on rings B and C. As anhydrotetracycline retained wild-type-like binding this change does not interfere with recognition. There are two possible explanations: (1) interactions with these functional groups are not energetically significant or (2) both functional groups act as H-bond acceptors and thus switching them does not prevent recognition. Noteworthy to mention that anhydrotetracycline changes ring B to an aromatic ring thus changing the overall shape and flexibility of the molecule but does not appear to have a significant effect on binding. Overall shape and flexibility often has a role in how a molecule accesses the binding site or whether it can access it at all. As riboswitches often fold around their ligands (binding site is created upon binding), flexibility might not be a significant issue.

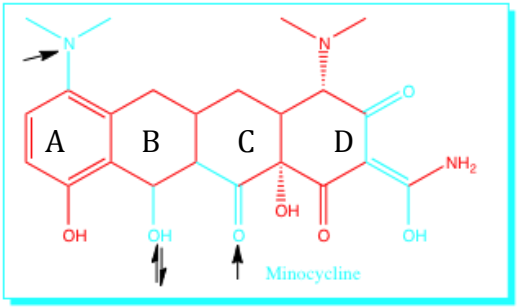
Oxytetracycline and doxycycline both appear not to bind. This result was most surprising, because these two derivatives were the most similar to tetracycline (Figure 20). Both of these derivatives alter the upper face of tetracycline. The major

difference between them and tetracycline is the addition of a hydroxyl group (circled) to ring C, which must affect the ability for recognition. This is likely due to either steric hindrance or hydrogen bonding to the tertiary amine that could act as a hydrogen bond acceptor. Testing a derivative with a methyl group attached to ring C might allow these two alternatives to be distinguished, but such derivative a is not commercially available. A hydroxyl group is also removed from ring B in doxycycline (boxed), but this modification is also present with anhydrotetracycline and it did not change the binding affinity and hence was deemed not significant.

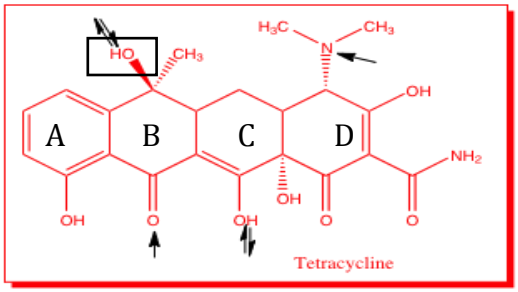
Derivatives that retained binding

Upper face





Lower face



Derivatives that failed to bind

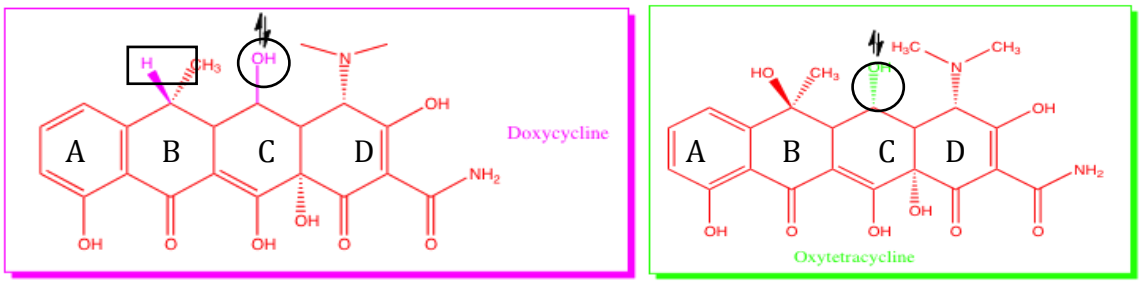


Figure 20. Tetracycline derivatives tested. Double arrows show a functional group that can be both H-bond acceptor and donor whereas single arrows function groups that act as H-bond acceptors.

Discussion

All assays need to be repeated using the new protocol. There were many alterations to the protocols to ensure that the binding affinities were most accurately determined. Purification was done on a small gel instead of a large urea gel. This was done to prevent the RNA from being lost or smeared as can be seen in Figure 30. Doing gel purification on a small gel provided a clean enough sample for the assays and reduced purification time from one day to a few hours. Considering the large number of mutants that need testing this time reduction is very significant. The original incubation period was 2 days; however, the assay results were better read when the incubation period was a minimum of 3 days. Because binding affinity is equilibrium dissociation constant it is essential that equilibrium is reached before the assays are read. This is determined via a trial and error process. Binding assays are read 30 minutes, 6 hours, overnight and few days past setup. The incubation time that gives the most consistent and tightest binding (smallest K_D value) is used in further characterizations. A shorter incubation time thus may result in the determination of a weaker apparent K_D value. Also included was RNase inhibitor to

prevent degradation of the RNA when the mixture was in the refrigerator for an extended length of time.

Some conclusions can be made regarding Krystal Roark's mutants, which were tested by her, Laura Edwards and in this study. Also a few conclusions may be determined on the new set of mutants as well as the tetracycline derivatives. All of Krystal Roark's mutants except 8 and 10 appear to be binding. Mutant 2 might have a 10-fold reduction in the K_D. Mutants 8 and 10 appear to not bind to the RNA under the assay conditions, but as it was only possible to go up to $2.0x10^{-6}$M RNA concentration (which is a rather high concentration considering a macromolecule) it is more accurately said that binding affinity is greater than about 500 nM, which is a significant decrease (50fold). The binding assays show that the K_D had the largest change when the mutations were made on the right side of the aptamer between nucleotides 41 and 50 (Figure 19).

(a) Development of a New Assay Protocol to Determine Binding Affinity of Loose Binder Mutants

The next step is to perform additional assays with the new protocol and to accurately determine the binding affinity of mutants that bind weakly to tetracycline. The problem with using this assay to determine binding affinity of mutants with a high K_D value (loose binding) is that it requires a lot of RNA. The RNA concentration needs to be at least 10x the K_D value. If the K_D is about 100 nM

the RNA has to be 1 μM, which is difficult to make. The binding affinity of mutants with a low K_D value (tight binding) can be easily determined with this assay, because it requires less RNA: if the K_D=1-10nM it is sufficient to use a few 100nM maximum RNA concentration. Due to the easiness of the fluorescent quenching assays and its high throughput nature it will continue to be used for initial screening and to determine binding affinity of mutants that bind well to tetracycline. To determine binding affinity of weak binders a few different options are available.

Fluorescent energy transfer can be used, which involves having donor and acceptor fluorephores. The donor emission will overlap with the acceptor excitation. If binding occurs, the donor will excite the acceptor. The RNA will be labeled with Rhodamine and act as the acceptor. The excitation will overlap with the tetracycline emission, which is acting as the donor. In this assay the RNA can be used at limiting concentrations while titrating in tetracycline. This will allow the use of less RNA to be needed, but more tetracycline, which is more readily available. This assay is still amenable to high throughput setup, but requires very high levels of incorporation of rhodamine fluorophore into the RNA. As the riboswitch RNA is too large for in vitro synthesis of labeled RNA (it is typically available for only up to 50 nucleotides), labeled RNA has to be created using in vitro transcription utilizing labeled nucleotides or enzymatic end labeling of RNA (phosphatase treatment to remove the terminal phosphate and kinase treatment using labeled nucleotide and in vitro transcribed RNA to introduce the fluorophore). These methods often do not provide

high enough incorporation of fluorophore label. This method was tried but unfortunately sufficient label incorporation was not achieved.

Another option is to use microcalorimetry. In this method the heat capacity of binding would have to be determined to find the K_D value. Unfortunately the calorimeter in the department in not suited for biochemical usage (requires too much material) and thus microcalorimetry is not a feasible method, unless new equipment is acquired.

The last option is to use a method that is not solution based. This would provide the added advantage of measuring binding affinities in a fundamentally different way that would provide a nice control. Krystal Roark and Alysa Frank developed nuclease protection assays that could be used to measure binding affinity of weak binders and to recheck the binding affinity of the wild type RNA as a control. In this assay the 5′ end of the RNA is labeled. The RNA is then subjected to nuclease cleavage without tetracycline and with increasing amounts of tetracycline. Then cleavage products are resolved on a denaturing gel. The cleavage patterns are different in the presence of tetracycline as tetracycline binds to the RNA and protects nucleotides from cleavage by the nuclease. As the tetracycline is added gradually the pattern equivalently would change allowing the determination of the K_D value. Even though this assay is low throughput it would only need to be used for the new mutants making these experiments manageable.

Thus the best assay for future studies is using fluorescent quenching for initial screening and to determine binding affinities of tight binders and using nuclease protection assays to determine the binding affinity of lose bindings.

Analysis of Tetracycline Analogues and Sensor Mutants *In Vivo*

It is necessary to compare the results of the fluorescent quenching assays, which is an *in vitro* study with *in vivo* data. In case of *in vitro* binding assays accurate results can be obtained about the binding affinity, but since cellular factors needed for transcription and translation are not present these assays test binding only and not how a given nucleotide or functional group contributes to the overall function of the riboswitch. A mutant/derivative that does not bind to tetracycline is not expected to be functional *in vivo* either, but a mutant/derivative that retains binding may be inactive *in vivo* due to interference with transcription or translation. Recall that the ykkCD riboswitch has been hypothesized to specifically recognize tetracycline and undergo a conformational change. This triggers production of the ykkCD efflux pump that in turn removes tetracycline toxic to the cell. To test how tetracycline derivatives and conserved riboswitch nucleotides contribute to riboswitch function *in vivo* we set up two different procedures. (1) To evaluate the amount of ykkCD pump mRNA *Bacillus Subtilis* cells will be grown in the presence and absence of tetracycline and its derivatives, and ykkCD pump mRNA levels will be quantified using quantitative real-time PCR. Nick Frecker in our lab is currently

testing tetracycline derivatives for this purpose. This method did not require cloning or alteration of *B Subtilis* genome but uses the assumption that mRNA levels correlate well with protein levels, which is not always the case. (2) Thus the amount of ykkCD pump protein produced in the presence of tetracycline derivatives also will be quantified. However quantifying the level of a particular protein in the cell is difficult in the absence of a good antibody against the protein. Thus a construct was created where the riboswitch regulates the expression of the β-galactosidase gene in a shuffle vector that works both in Gramm negative (*E Coli*) and Gramm positive bacteria (*B Subtilis*). The "shuffle" nature of this construct allowed cloning to be done in *E Coli* where it is much more easily done and assays to be conducted in *B. subtilus*. This construct is not designed to replace the ykkCD efflux pump in *B Subtilis* genome (if the efflux pump were removed, cells would be expected to die in the presence of high tetracycline concentrations); it is designed to replace the nonessential Amy gene in *Bacillus* thus the construct is replicated with the Bacillus genome (Figure 21). Bacillus cells will be grown with tetracycline and its derivatives and the β- galactosidase enzymatic activity will be measured using a colorimetric assay to evaluate the amount of protein production. This is a widely used technique to determine regulation of an essential gene. In case of derivatives that failed to bind tetracycline we expect low pump mRNA levels and low β-galactosidase production. In case of derivatives that retained binding an increase in pump mRNA levels and high β-galactosidase activity is expected. These expectations are consistent with the

hypothesis where the riboswitch recognizes tetracycline and triggers efflux pump production that in turn removes the toxic molecule allowing survival of the cells.

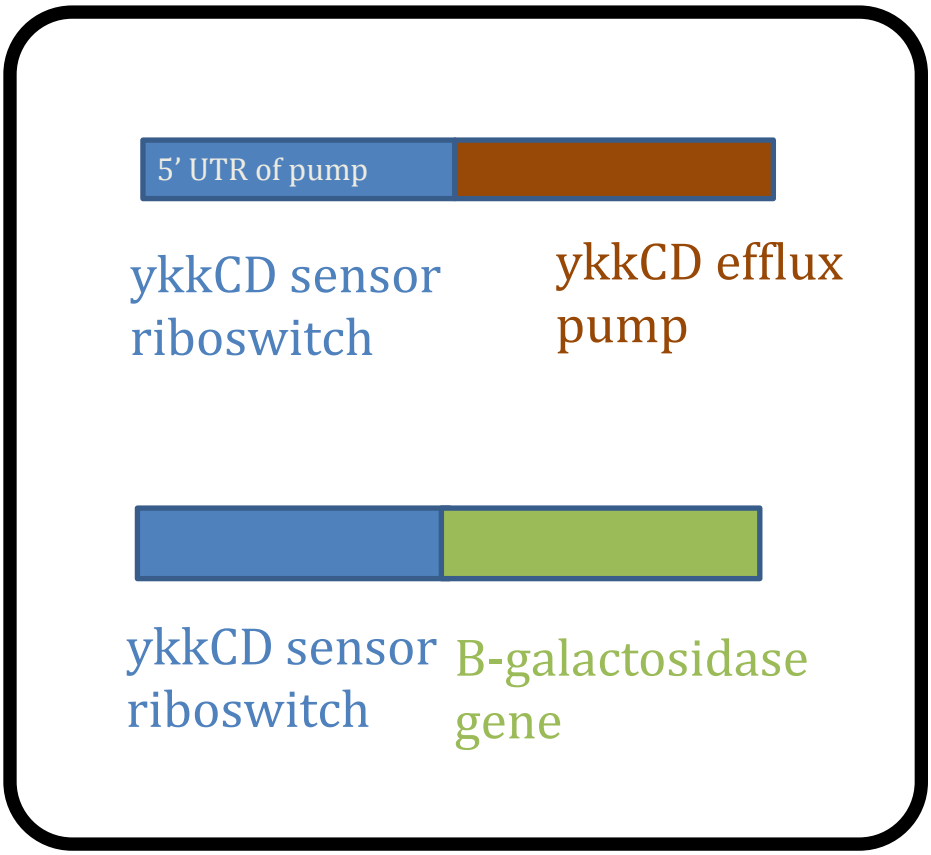


Figure 21 Quantifying protein levels in vivo.

Appendix

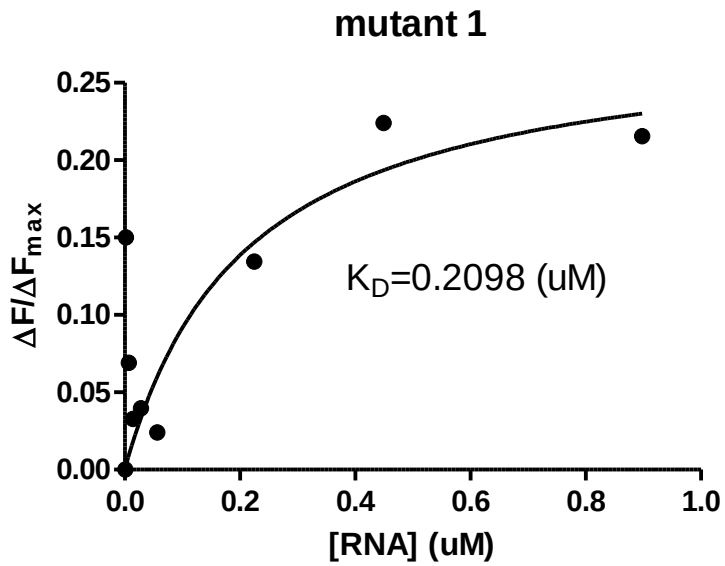
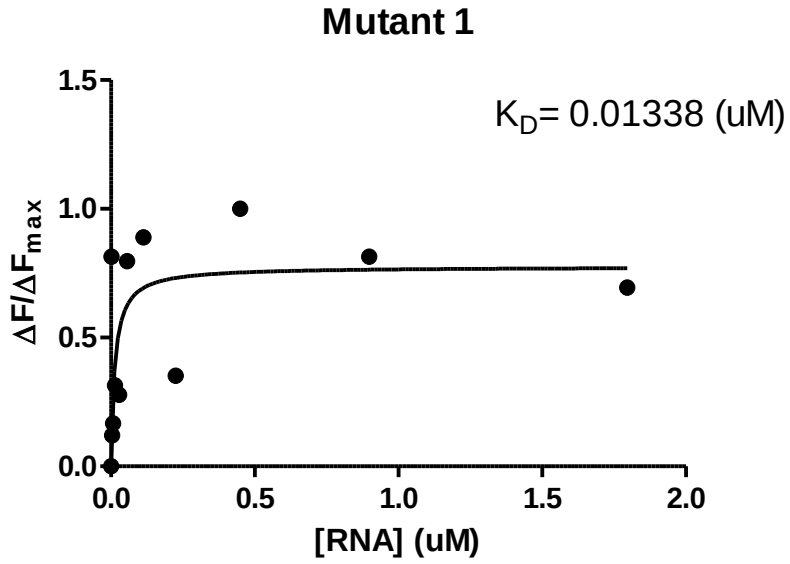


Figure A.1 & A2 Mutant 1 binding assays.



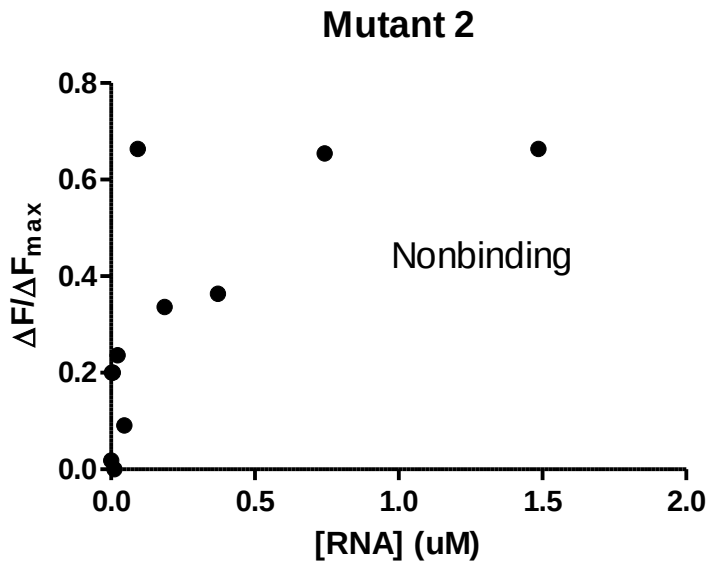


Figure A.3 Mutant 2 binding assay.

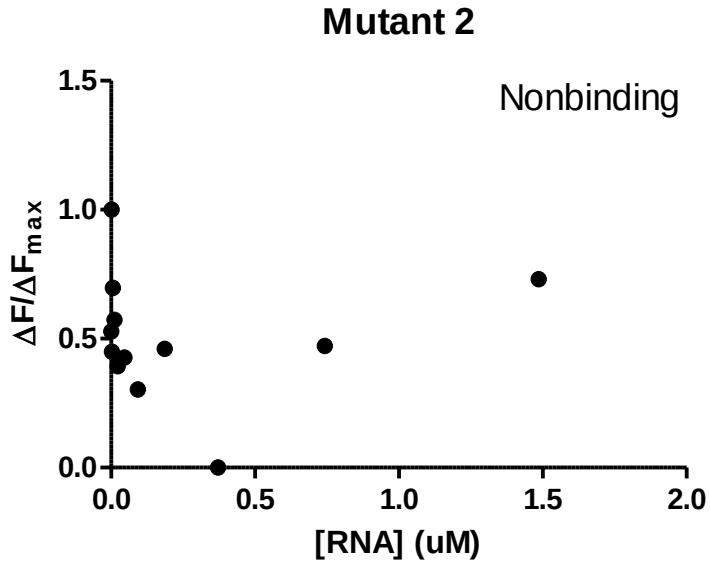


Figure A.4 Mutant 2 binding assay.

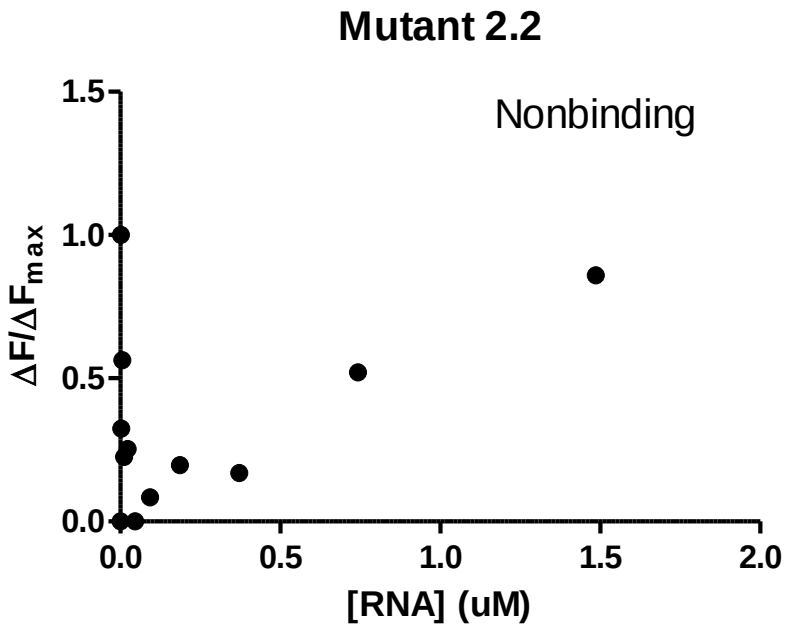


Figure A.5 Mutant 2 binding assay.

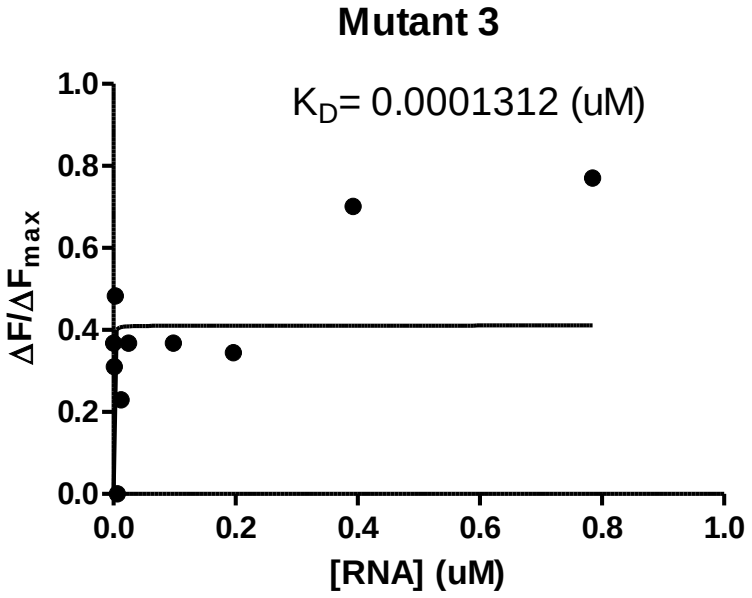


Figure A.6 Mutant 3 binding assay.

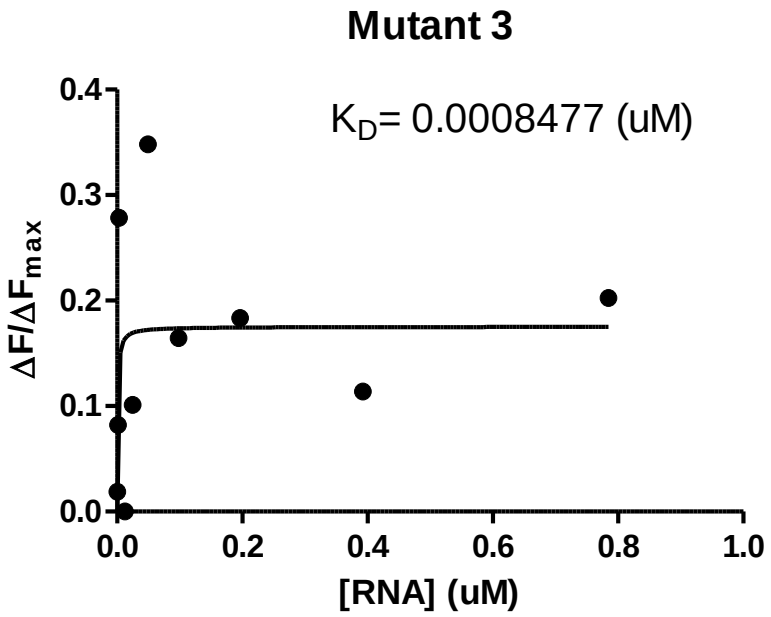


Figure A.7 Mutant 3 binding assay.

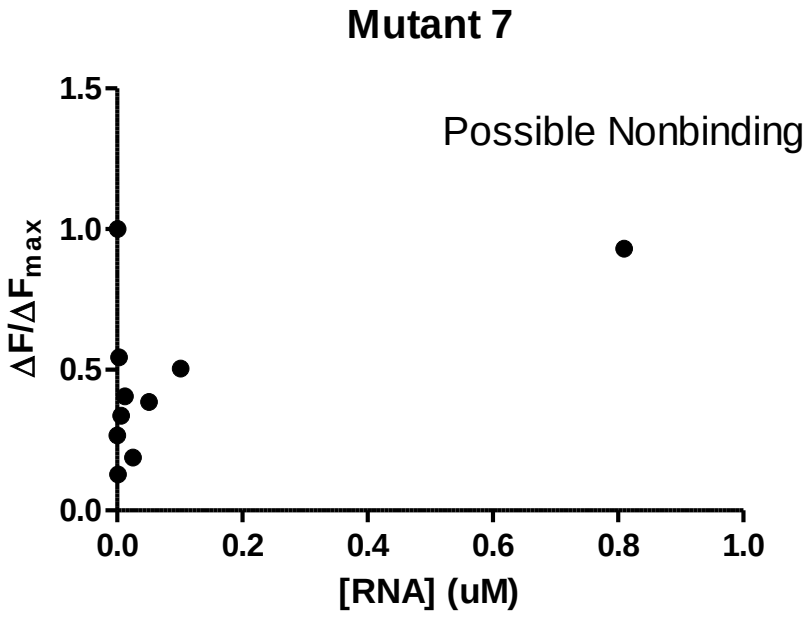


Figure A.8 Mutant 7 binding assay.

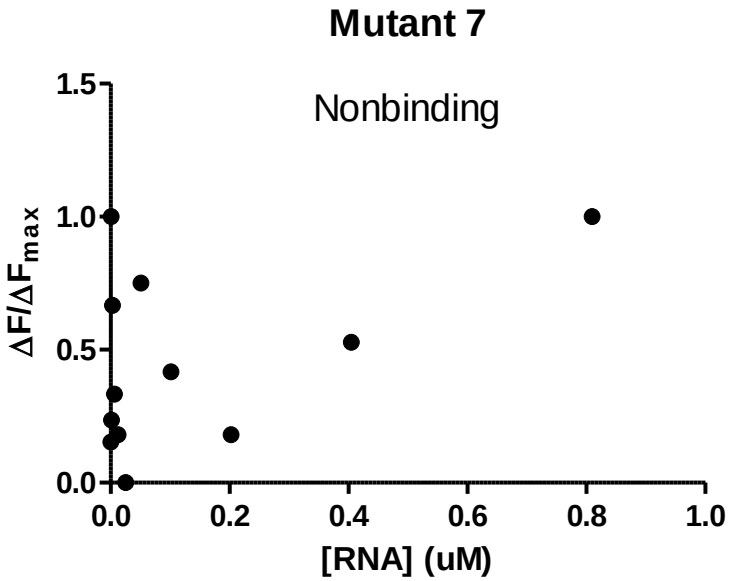


Figure A.9 Mutant 7 binding assay.

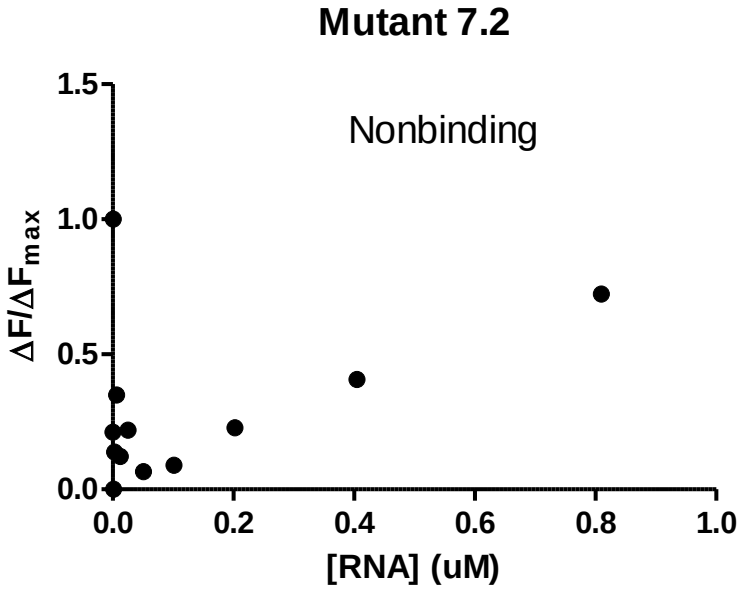


Figure A.10 Mutant 7 binding assay.

Mutant code	Primer seqeunceas	Bases	Sequence	Molecular Weight	Tm (50mM NaCl) C
1	1top	41	GTC AAT TGA CAT GGA CTG GTG GGA GAG AAA ACA CAT ACG CG	12756.3	80.15833659
1	1bottom	41	CGC GTA TGT GTT TTC TCT CCC ACC AGT CCA TGT CAA TTG AC	12453.1	80.15833659
2	2top	38	TGT AAA GTT TTC TAT TCC GCA TGT CAA TTG ACA TGG AC	11657.6	70.94650526
2	2bottom	38	GTC CAT GTC AAT TGA CAT GCG GAA TAG AAA ACT TTA CA	11693.7	70.94650526
3	3top	45	GTA AAG TTT TCT ACC CTT CCG CAT GTC AAT TGA CAT GGA CTG GTC	13777	78.05555556
3	3bottom	45	GAC CAG TCC ATG TCA ATT GAC ATG CGG AAG GGT AGA AAA CTT TAC	13902.1	78.05555556
4	4top	45	TGT AAA GGG GGC TAG GGT TCC GCA TGT CAA TTG ACA TGG ACT GGT	14012.1	78.56666667
4	4bottom	45	ACC AGT CCA TGT CAA TTG ACA TGC GGA ACC CTA GCC CCC TTT ACA	13669.9	78.56666667
5	5top	45	ATT GAC ATG GAC TGG TCC GTC TTT TGA CAT ACG CGT AAA TAG AAG	13899.1	63.81111111
5	5bottom	45	CTT CTA TTT ACG CGT ATG TCA AAA GAC GGA CCA GTC CAT GTC AAT	13779	63.81111111
6	6top	33	CGC ATA TGC ACA CGG TCC GAA AAA AGC CCG GGA	10150.6	63.43939394
6	6bottom	33	TCC CGG GCT TTT TTC GGA CCG TGT GCA TAT GCG	10118.6	63.43939394
7	7top	24	CGG AGG GAA AAA AGC GGC GGA GAG	7575	66.5
7	7bottom	24	CTC TCC GCC GCT TTT TTC CCT CCG	7133.6	66.5

Table 2. Primer sequences for second set of mutations.

Mutant	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Trial 7	Trial 8	Trial 9	Trial 10
1.	25.000	20.000	9.900	83.770						
2.	1.822	31.320	10.760	23.000	39.49	31.32	10.76	1.820		
3.	3.464	3.447	1.244		11.00	12.00	1.24	1.059	3.4	3.4
4.										
5.	6.510	1.362	2.100	2.200	1.00	0.95	2.80	1.360		
6.	68.850	26.830	267.410	8.240	95.26					
7.	3.563	5.350	8.356	5.356	8.10					
8.										
9.	4.456	7.202	4.000	2.480	4.40	7.20				
10.										
11.	*248.000**	9.900	2.480	6.700	5.20	3.40				

Table 3. Krystal Roark Mutant's K_D Values

Mutants	Trial 1	Trial 2	Trial 3
1.	0.2098	0.01338	0.324
2.			
3.	40.8400	38.47000	
4.			
5.			
6.			
7.			

Table 4. Delores Mutant's K_D value

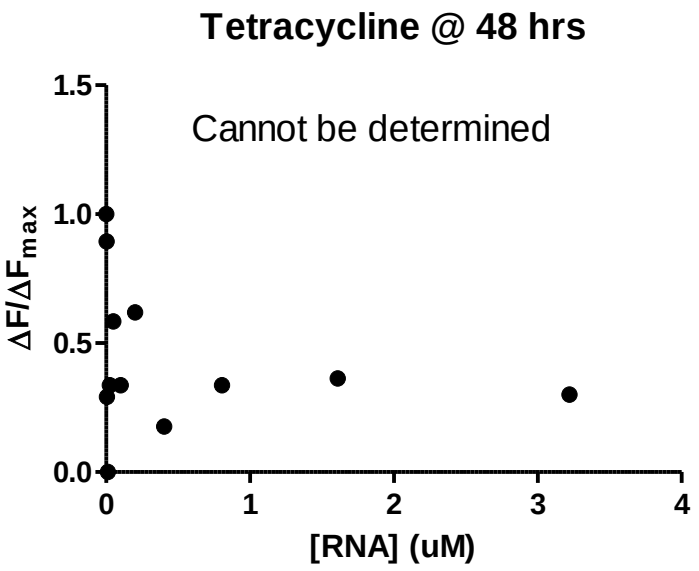


Figure A. 11 Tetracycline binding assay

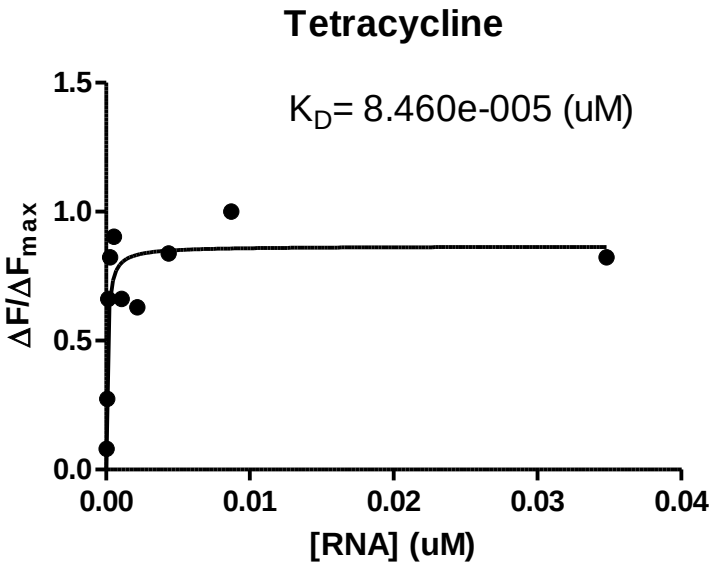


Figure A. 12 Tetracycline binding assay

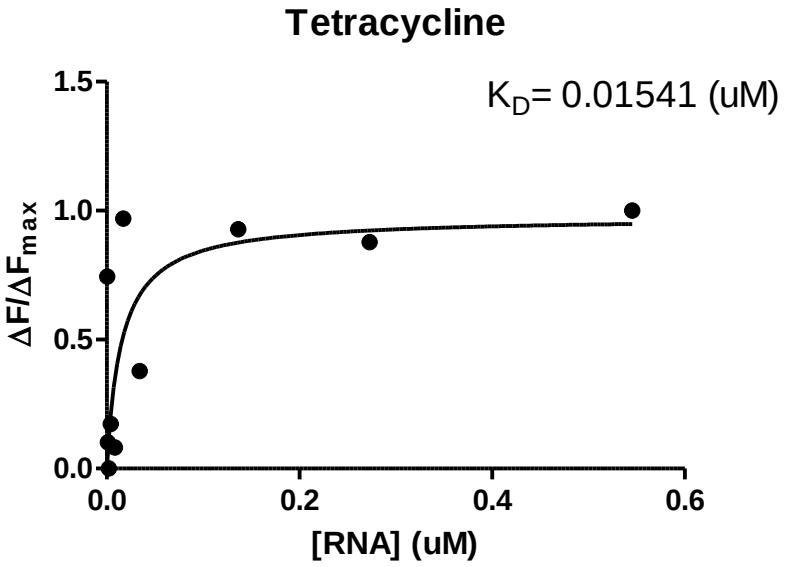


Figure A. 13 Tetracycline binding assay

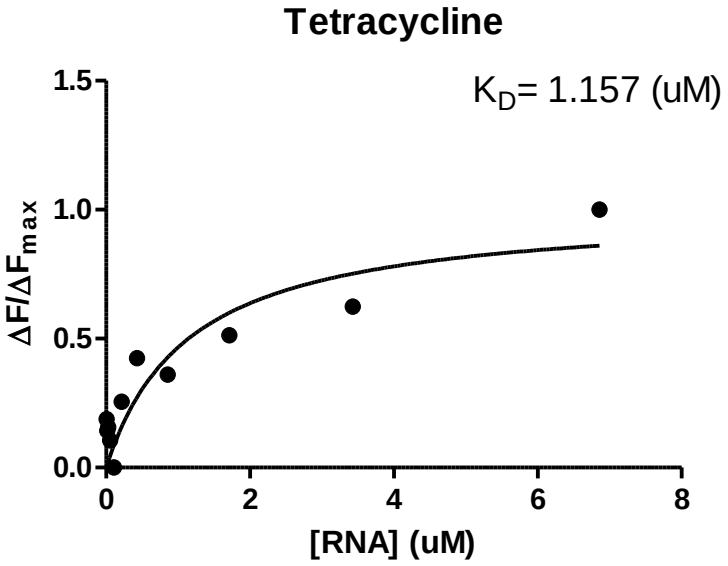


Figure A. 14 Tetracycline binding assay

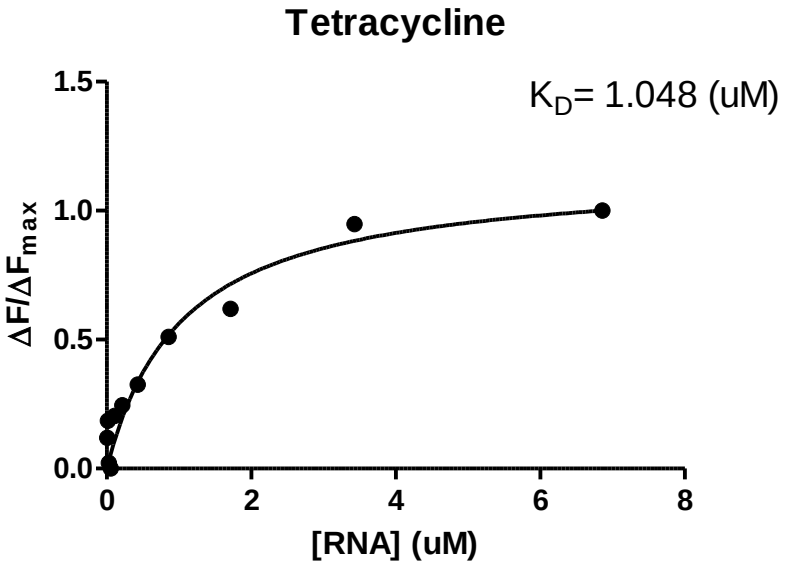


Figure A. 15 Tetracycline binding assay

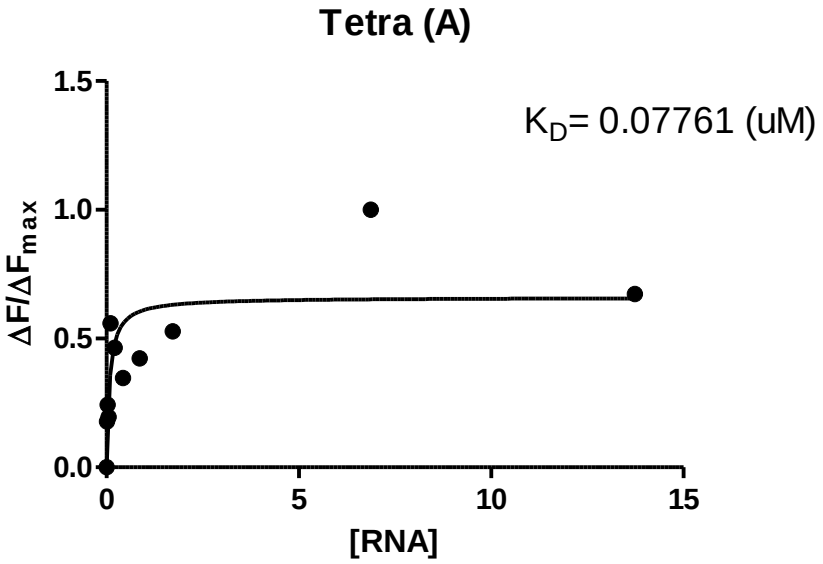


Figure A. 16 Tetracycline binding assay

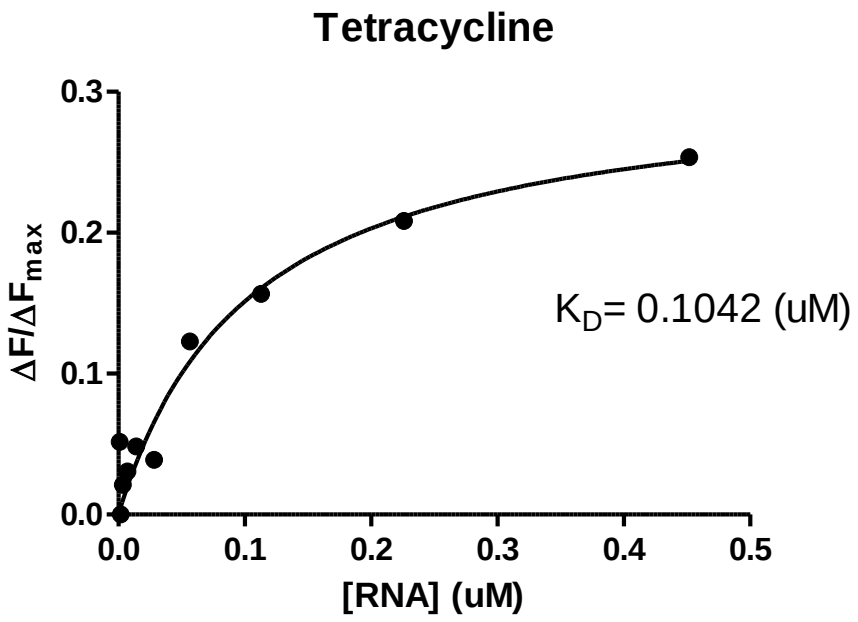


Figure A. 17 Tetracycline binding assay

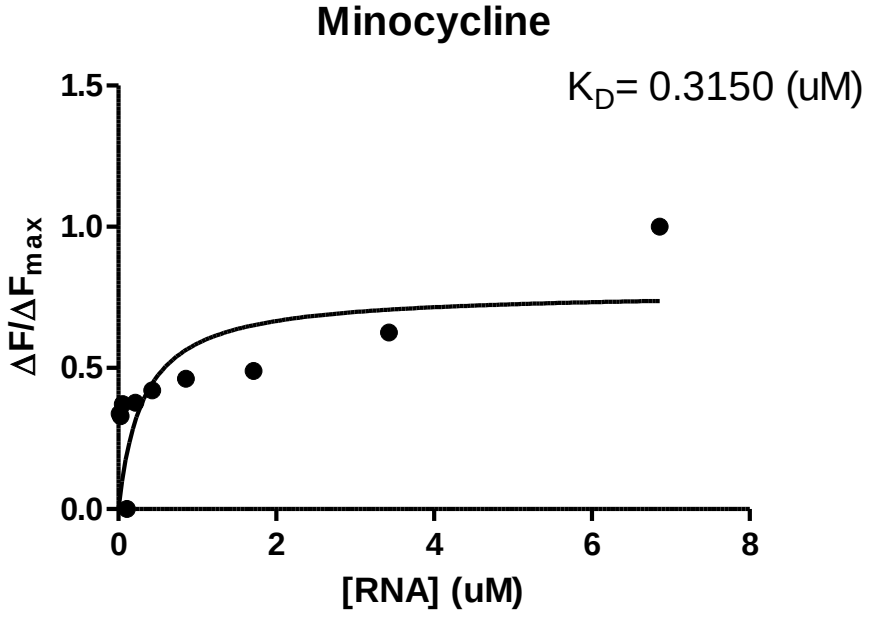


Figure A. 18 Minocycline binding assay

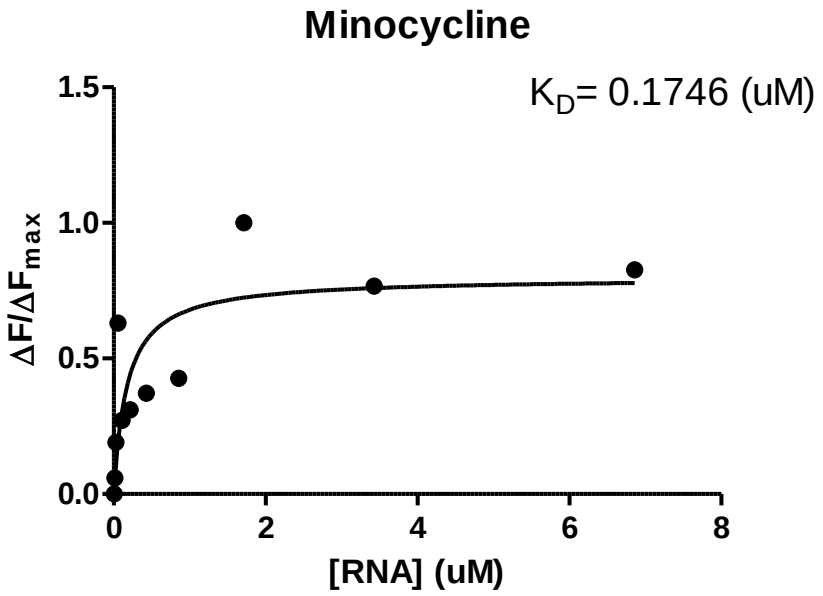


Figure A. 19 Minocycline binding assay

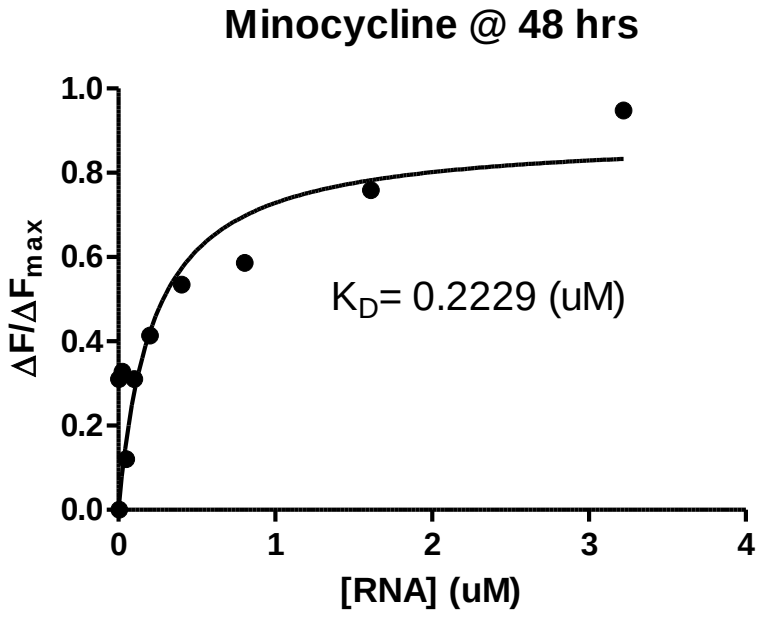


Figure A. 20 Minocycline binding assay

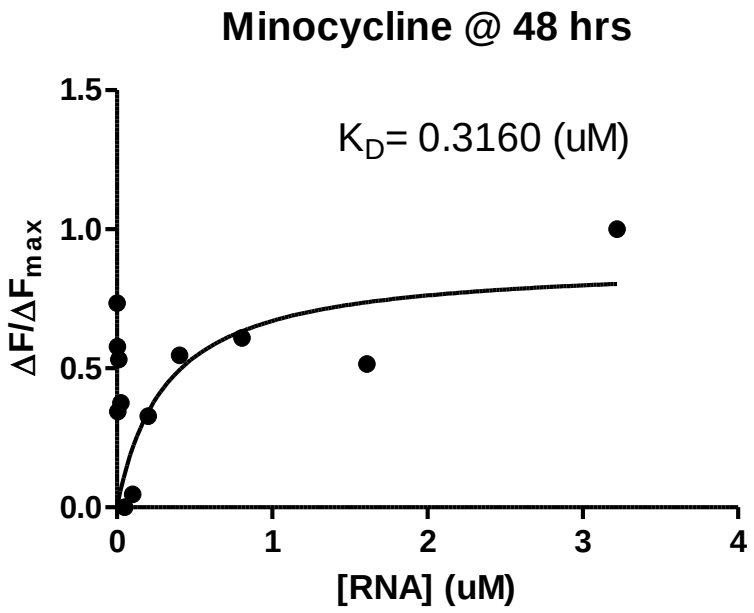


Figure A. 21 Minocycline binding assay

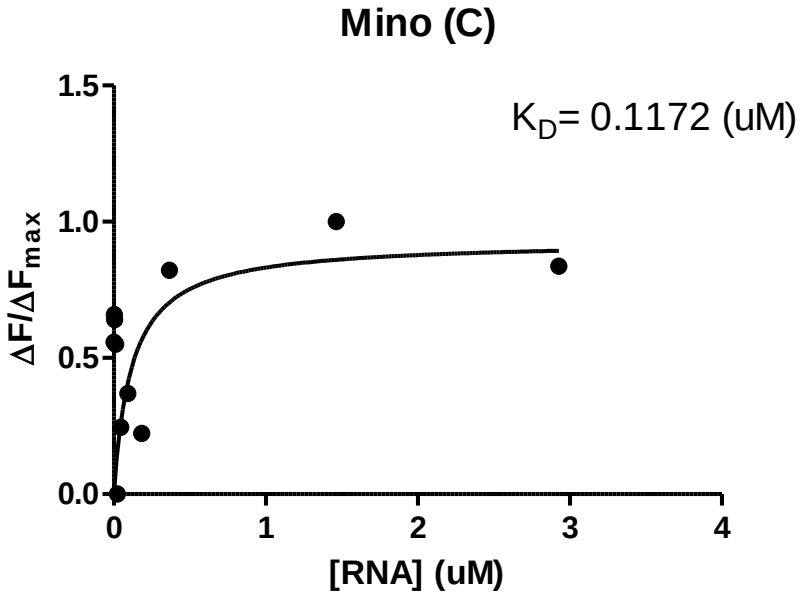


Figure A. 22 Minocycline binding assay

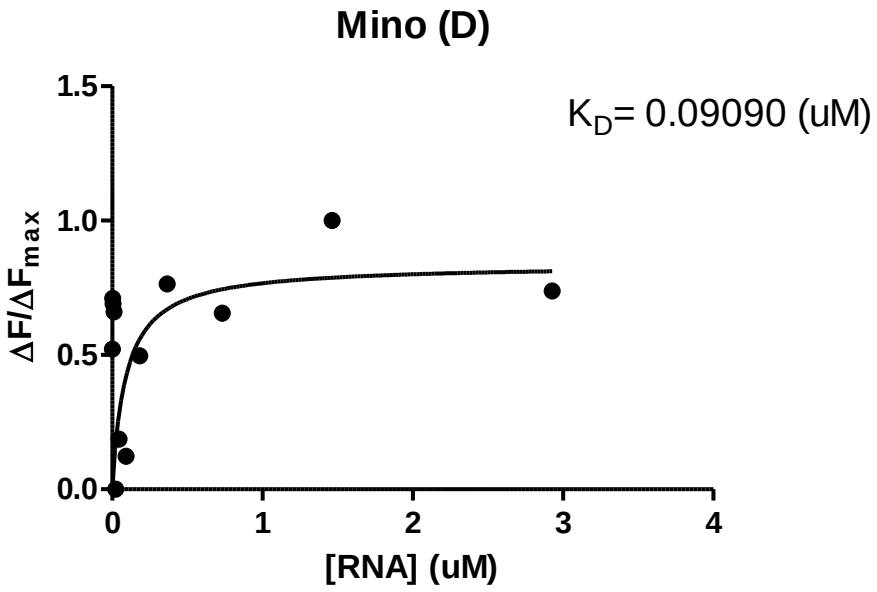


Figure A. 23 Minocycline binding assay

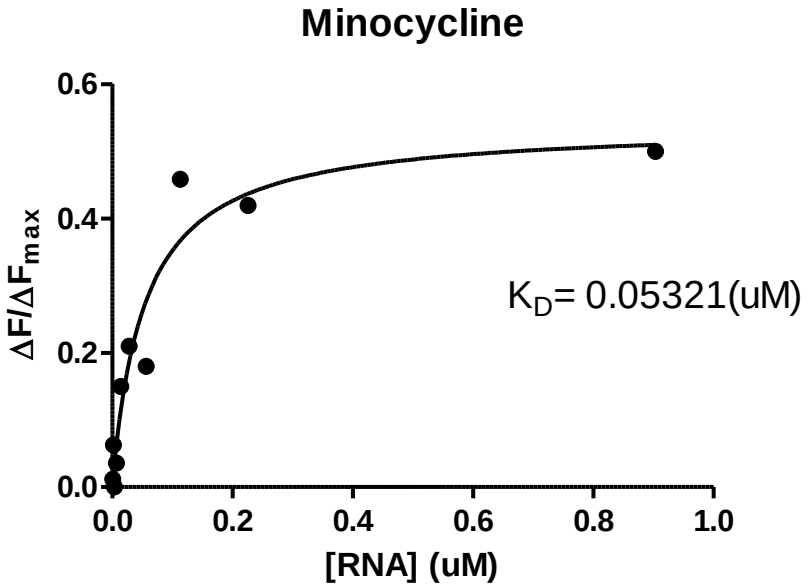


Figure A. 24 Minocycline binding assay

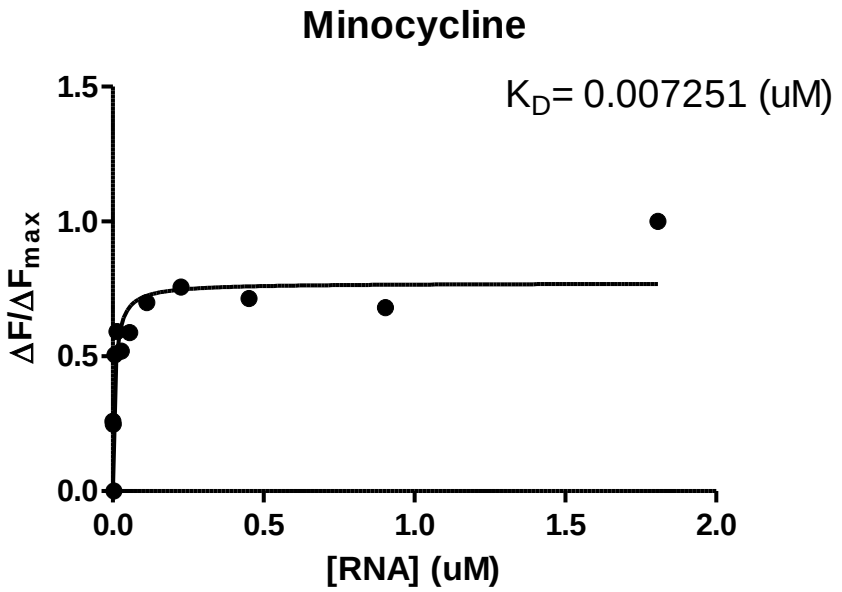


Figure A. 25 Minocycline binding assay

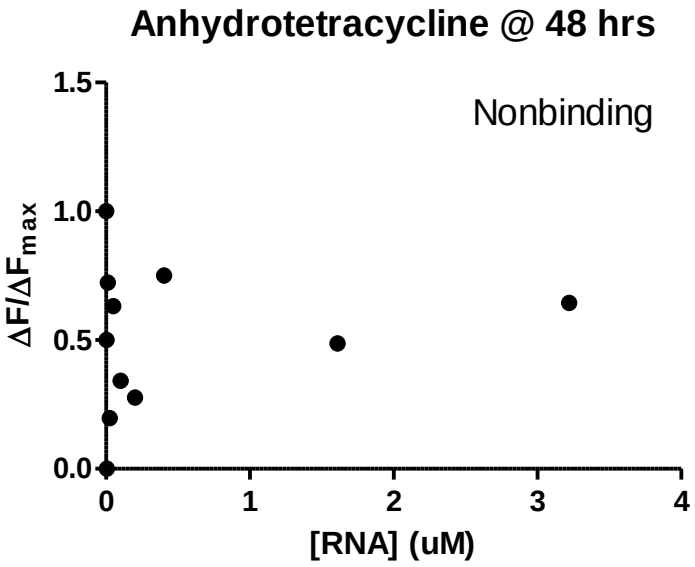


Figure A. 26 Anhydotetracycline binding assay

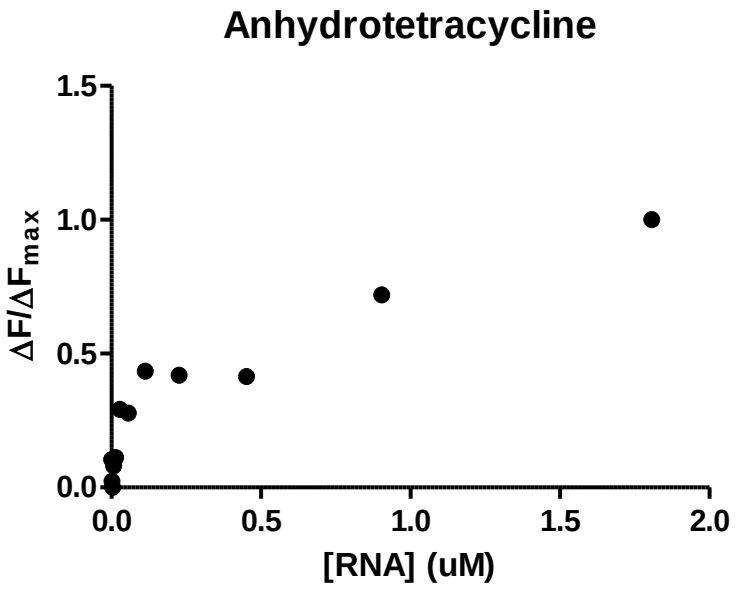


Figure A. 27 Anhydotetracycline binding assay

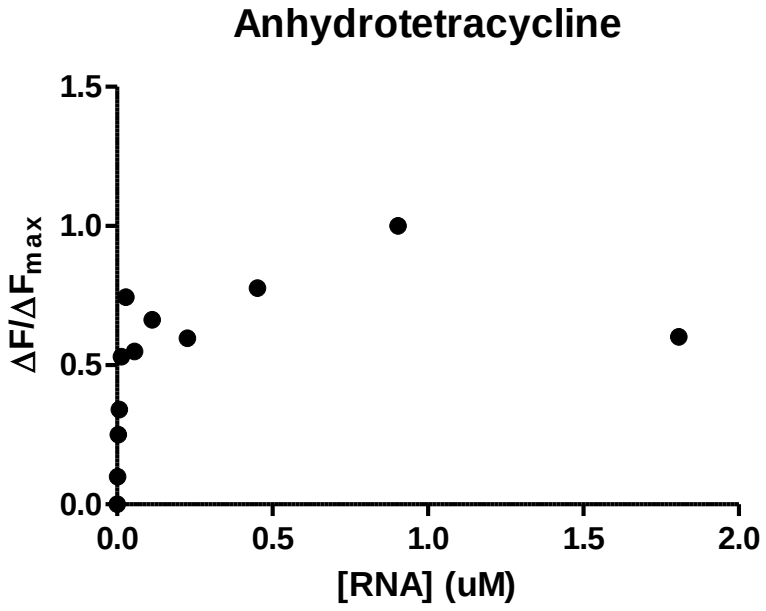


Figure A. 28 Anhydotetracycline binding assay

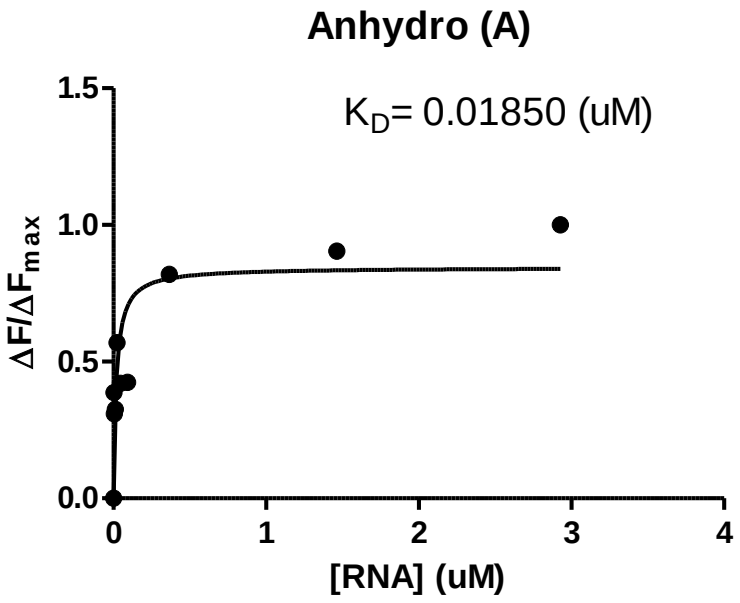


Figure A. 29 Anhydotetracycline binding assay

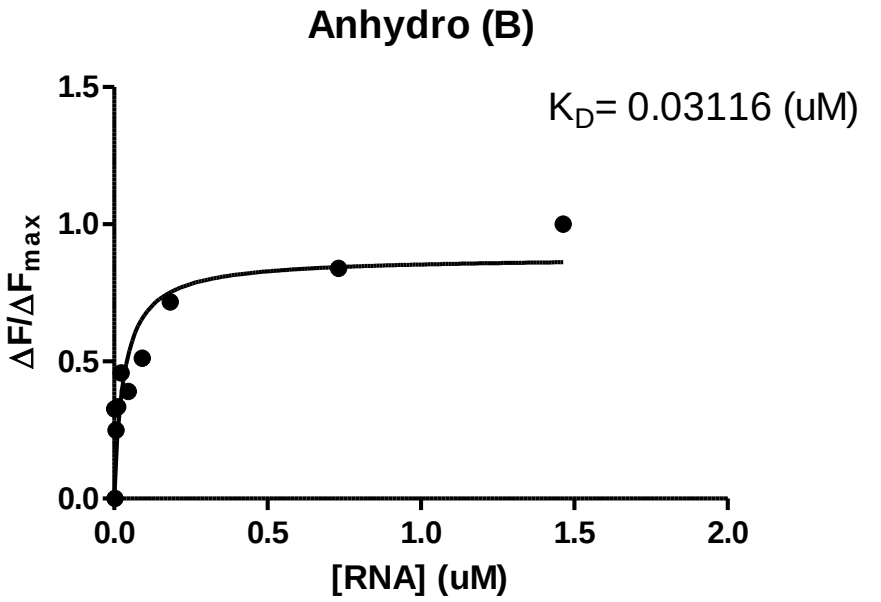


Figure A. 30 Anhydotetracycline binding assay

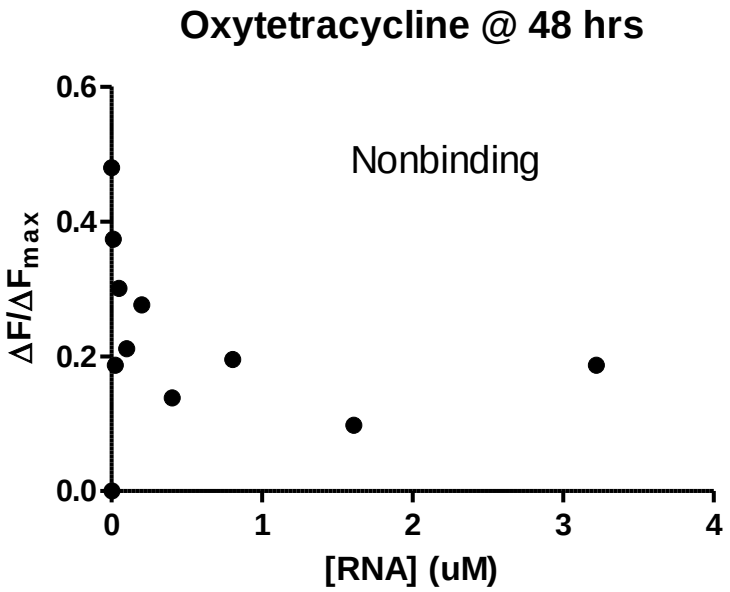


Figure A. 31 Oxytetracycline binding assay

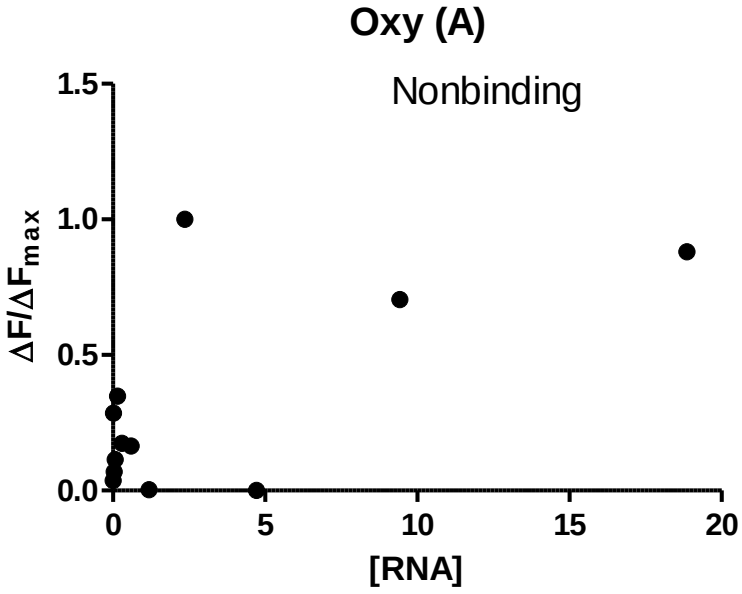


Figure A. 32 Oxytetracycline binding assay

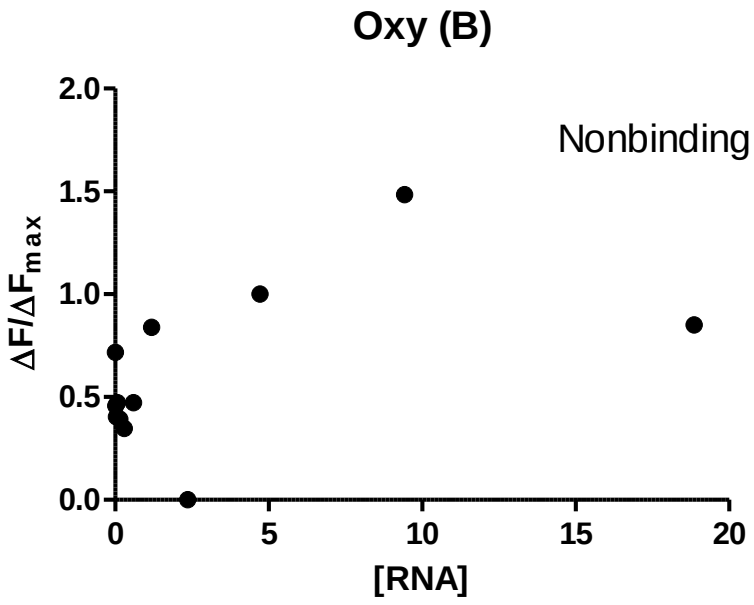


Figure A. 33 Oxytetracycline binding assay

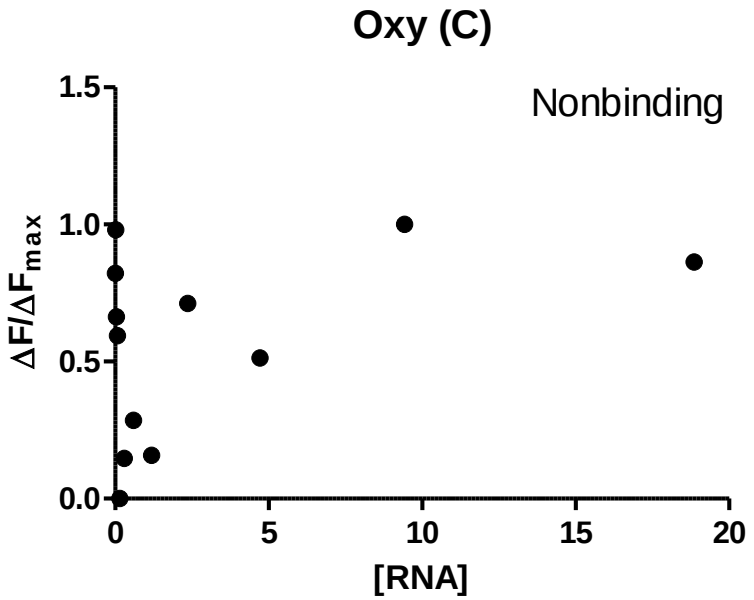


Figure A. 34 Oxytetracycline binding assay

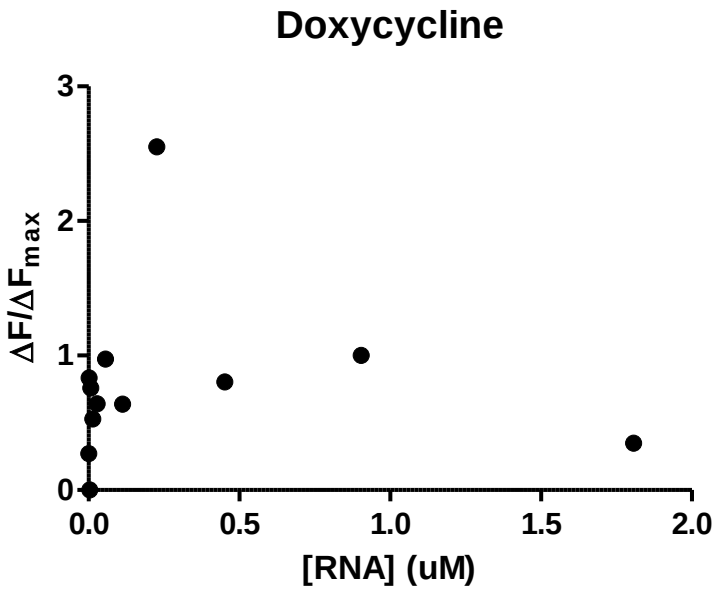


Figure A. 35 Doxycycline binding assay

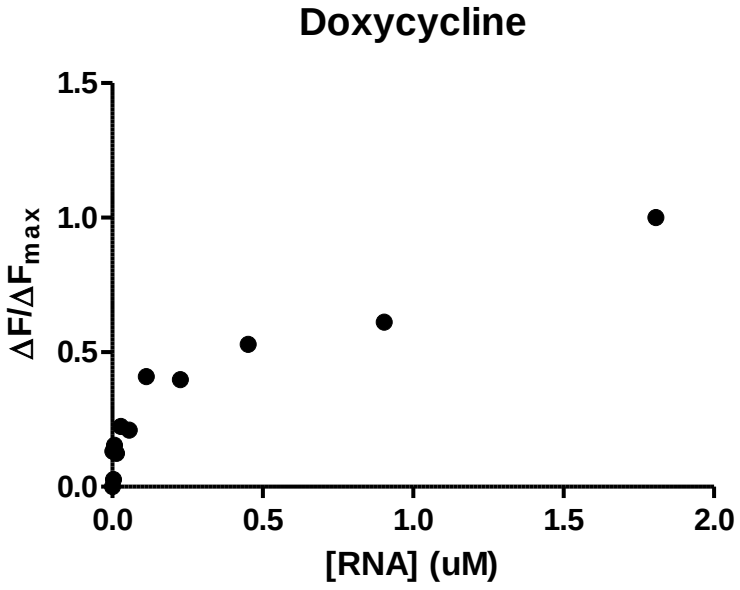


Figure A. 36 Doxycycline binding assay

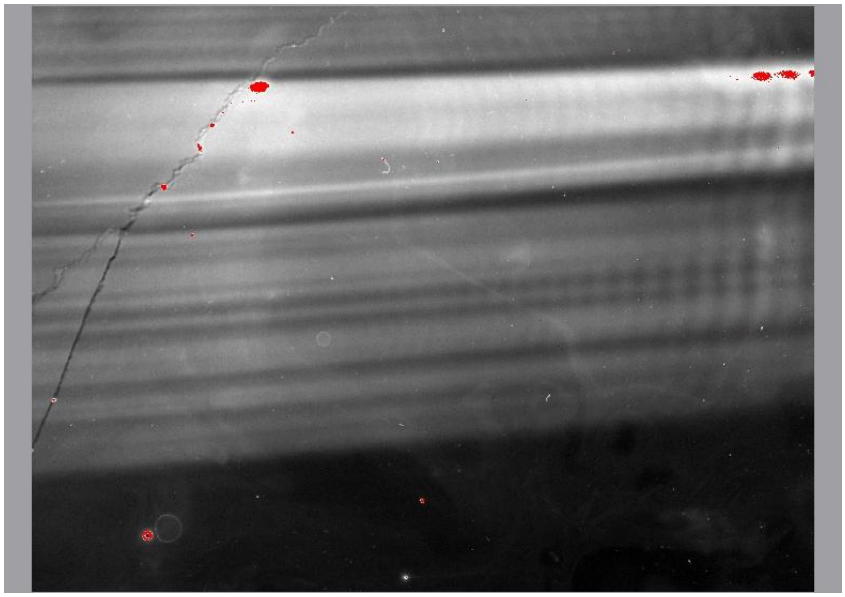


Figure A. 37 Gel purification exemplifying the RNA being smeared across the large urea gel.

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